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Universidad del País Vasco
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Sediment as tool for evaluating ecological status of a river basin

Physicochemical and biological quality indicators

El sedimento como herramienta evaluadora del estado ecológico de una cuenca hidrográfica

Indicadores de calidad fisicoquímica y biológica

Memoria de tesis doctoral

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Presenta la siguiente memoria de tesis para optar al grado de Doctora en Ingeniería Ambiental por la Universidad del País Vasco - Euskal Herriko Unibertsitatea (UPV-EHU):

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A mi ama,

por permanecer siempre a mi lado.

A mi aita,

por permanecer siempre en mi memoria.

RESUMEN

La continua intensificación de la industria vasca ha permitido fortalecer el tejido empresarial en el que se sostiene una población en constante crecimiento. Sin embargo, este hecho ha generado múltiples presiones sobre el medio ambiente, llegando a comprometer el estado ecológico de los ríos, a lo largo de los cuales se asientan los mayores núcleos urbanos e industriales. En esta coyuntura se hace imprescindible la consecución de una armonización entre la protección del medio ambiente y un desarrollo regional sostenible. Con este propósito, se han acometido recientemente cambios en la gestión de las aguas residuales urbanas e industriales en la cuenca del río Deba (Gipuzkoa), una de las más contaminadas del País Vasco. A la degradación de la calidad de sus aguas, hay que añadir el impacto que dichos vertidos ocasionan en la calidad de los sedimentos, que actúan como fuente, sumidero y vector de transporte de contaminantes. Sin embargo, a pesar de que proporciona un registro histórico de la contaminación, la legislación europea, estatal y autonómica no otorga al Sedimento el mismo nivel de relevancia que al Agua en la evaluación del estado ecológico de las cuencas hidrográficas.

El objetivo principal de este trabajo fue remarcar el papel fundamental de los sedimentos en la dinámica de contaminantes en los ecosistemas acuáticos, y valorar el efecto de la gestión de vertidos de aguas residuales urbano-industriales sobre el estado ecológico de la cuenca del río Deba, empleando indicadores de calidad fisicoquímica, así como biológica.

A pesar de la implantación de medidas correctoras en esta zona de estudio, la evaluación temporal (de enero del 2015 a enero del 2016) de la contaminación a lo largo del cauce principal y los afluentes de la cuenca del río Deba ha constatado que los efluentes de las estaciones depuradoras y, muy especialmente, los vertidos de aguas residuales urbano-industriales sin tratar aumentan el contenido en materia orgánica, nutrientes y metales, tanto del agua como del sedimento. Además, la evaluación espacial conjunta de la calidad química, fisicoquímica y biológica de los sedimentos (campaña de muestreo de octubre del 2015) ha permitido corroborar el mal estado ecológico actual de algunas masas de agua; concretamente, el tramo medio-bajo del cauce principal y, primordialmente, el afluente Ego. Por tanto, este trabajo ha demostrado que, al menos, un muestreo y análisis anual del sedimento aporta una valiosa información prospectiva sobre el estado y evolución de la calidad de las aguas en los ríos,

identificando aquellas zonas susceptibles de polución y posibilitando la definición de nuevas estrategias futuras de sostenibilidad.

Adicionalmente, se ha observado que la combinación de dos técnicas de biología molecular para analizar la abundancia y biodiversidad (funcional y estructural) de las comunidades bacterianas presentes en los sedimentos, como son la reacción en cadena de la polimerasa cuantitativa (en inglés, quantitative polymerase chain reaction (qPCR)) y el metabarcoding del gen 16S ARNr (en inglés, rRNA), es un procedimiento adecuado para predecir cambios en las funciones ecosistémicas de los ríos y analizar su vulnerabilidad. En efecto, se ha demostrado que los vertidos de aguas residuales tratadas y no tratadas alteran la distribución espacial y composición de las comunidades bacterianas involucradas en los ciclos biogeoquímicos del nitrógeno y el azufre, lo que conduce a una posible emisión de gases de efecto invernadero (N_2O) como producto de una desnitrificación incompleta. Conjuntamente, la medición de la actividad enzimática (Nitrato Reductasa y Nitrito Reductasa) ha permitido evaluar el potencial desnitrificante de la comunidad microbiana presente en los sedimentos. Se ha comprobado que la actividad microbiana desnitrificante mitiga parcialmente el alto contenido en nitratos en los sedimentos de algunos tramos del río y que, por tanto, podría no ser suficiente para soportar las altas cargas de nutrientes y garantizar la sostenibilidad de la cuenca.

Por otra parte, la determinación conjunta del contenido total, especiación química y bioaccesibilidad de los metales como indicadores químicos de la potencialidad tóxica de los sedimentos ha permitido detectar las fuentes antropogénicas de estas especies. Simultáneamente, se ha evaluado el riesgo que suponen tanto para la salud ecosistémica como humana con mayor exactitud y de forma más realista que las actuales directivas, que establecen las normas de calidad ambiental (NCA) en función, únicamente, de su contenido total. Este trabajo ha demostrado que la bioaccesibilidad y, por tanto, la toxicidad de los metales depende de su especiación química que obedece, a su vez, a la composición mineralógica y elemental del sedimento, siendo el tamaño de partícula un parámetro determinante.

Finalmente, el estudio de la influencia de la estacionalidad del régimen hidrológico sobre la migración de los metales asociados a las partículas en suspensión ha evidenciado un mayor riesgo ecosistémico y humano durante la época de estiaje debido, fundamentalmente,

a la resuspensión de las partículas más finas del sedimento, que albergan un mayor contenido de metales. Asimismo, se ha observado una ciclicidad en la función del sedimento, que pasa de ser fuente a sumidero de metales en condiciones de estiaje.

ABSTRACT

Continuous intensification of Basque industry has allowed to strengthen the business fabric, which sustains a constant growing population. Nevertheless, this fact has generated multiple pressures on the environment and, consequently, it compromises the ecological status of rivers, where the most important urban and industrial areas are concentrated. In this situation, balancing the environmental protection with a sustainable development of the region becomes an essential goal. With this purpose, recent changes have been made in the management of urban and industrial wastewaters in the Deba River (Gipuzkoa), one of the most polluted catchments in the Basque Country. In addition to the degradation of water quality, the impact of wastewater discharges on sediment quality should be considered. Indeed, Sediment act as source, sink and transport vector of pollutants. However, despite it provides a history of contamination, European, State and Autonomous Community legislation does not give to Sediment the same level of relevance than to Water for assessing the ecological status of river basins.

The main objective of this work was to emphasize the key role of sediments in pollutant dynamics in aquatic ecosystems, and to evaluate the influence of urban and industrial wastewaters management on the ecological status of the Deba River catchment, using both physicochemical and biological quality indicators.

During the study of the temporal (sampling campaigns from January 2015 to January 2016) and spatial evolution of contamination in such area, we found that, despite the implementation of corrective actions, effluents from the wastewater treatment plants and, mainly, untreated urban and industrial wastewater discharges increased organic matter, nutrient and metal content in water and sediments from the main channel and the tributaries of the Deba River catchment. In addition, the joint spatial evaluation of the chemical, physicochemical and biological quality of sediments (sampling campaign of October 2015)

confirmed the current poor ecological status of some water bodies; specifically, the mid-low part of the main river and, primarily, the Ego stream. Therefore, this work demonstrated that, at least, an annual sediment sampling provides a valuable information for prospective monitoring water quality, identifying those sites which could deserve special attention for planning future sustainability strategies.

Additionally, we observed that the combination of functional qPCR and structural (16S RNA gene) metabarcoding is a suitable approach to predict changes in river ecosystem functions, and to evaluate the vulnerability of sediment bacterial community. Indeed, it was proved that treated and untreated wastewater discharges alter the spatial distribution and composition of bacterial communities involved in nitrogen and sulphur cycling, favoring possible greenhouse gas (N_2O) emissions as a result of an incomplete denitrification. Moreover, the potential of sediment bacterial community for denitrification was also evaluated by enzymatic activity measurement (Nitrate Reductase and Nitrite Reductase enzymes). We observed that the excess of nitrate present in sediments from some river sections is partially mitigated, and that denitrifying microbial activity might not be sufficient to support the high nutrient loadings and to ensure river sustainability.

On the other hand, the determination of metal total content, chemical distribution and bioaccessibility as chemical indicators of sediments allowed us to identify the anthropogenic sources of metals. In addition, the ecological and human health risk assessment based on metal bioaccessibility was more accurate and reliable than the application of environmental quality standards (EQS), which mean their total concentration in sediments. In fact, this work demonstrated that the bioaccessibility and, therefore, the toxicity of metals depends on their chemical distribution which, in turn, is subjected to the mineralogical and elemental composition of sediments, being the particle size distribution a key parameter.

Finally, studying the influence of hydrological regime seasonality on the migration of metals associated to suspended particulate matter suggested that low flow season poses the highest ecological and human health risk. This might be primarily due to the resuspension of fine sediments with significantly higher metal content than bulk sediments. Likewise, a cyclical behaviour of sediments was also observed since they act as sink for metals instead of metal source during dry periods.

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a.s.l.	At Sea Level
ADN	Ácido Desoxirribonucleico
AnAOB	Anaerobic Ammonia–Oxidizing Bacteria
ANOVA	Analysis of Variance
AOB	Ammonia–Oxidizing Bacteria
ARNr	Ácido Ribonucleico Ribosómico
B	Bioaccessibility/Bioaccesibilidad
BAER	Bioaccessibility Assessment Evaluation Ratio
BCR	Community Bureau of Reference
C	Concentration/Concentración
CDI	Chemical Daily Intake
CE	Conductividad Eléctrica
CFG	Condiciones Fisicoquímicas Generales
CFI	Cantabrian Fish Index
CP	Contribution Percentage
Ct	Cycle Threshold
d.l.	Detection Limit
DBO_5	Demanda Biológica de Oxígeno a 5 días
DDT	Dichlorodiphenyltrichloroethane
DF	Divergence Factor
DIN	Deutsches Institut für Normung–German Institute for Standardization
DMA	Directiva Marco del Agua
DNA	Deoxyribonucleic Acid
DNB	Denitrifying Bacteria
DNRA	Dissimilatory Nitrate Reduction to Ammonium
DOC	Dissolved Organic Carbon
DQO	Demanda Química de Oxígeno
DW	Dry Weight
EC	Electrical Conductivity
E_D	Exposure Duration
EDAR	Estación Depuradora de Aguas Residuales
EDTA	Ethilenediamine Tetraacetic Acid
EF	Enrichment Factor
E_F	Exposure Frequency
Eh	Redox Potential/Potencial Redox
EQR	Ecological Quality Ratio
EQS	Environmental Quality Standard

ERF	Ecological Risk Factor
ERL	Effect Range Low
ERM	Effect Range Median
ESP	Exchangeable Sodium Percentage
ETAP	Estación de Tratamiento de Agua Potable
GCF	Global Contamination Factor
GEF	Global Enrichment Factor
GIRH	Gestión Integrada de Recursos Hídricos
HQ	Hazard Quotient
IB	Indicador Biológico
IBMR	Índice Biológico de Macrófitos en Ríos
ICF	Individual Contamination Factor
ICP–OES	Inductively Coupled Plasma–Optical Emission Spectrometer
IFQ–R	Índice Fisicoquímico Referenciado
Igeo	Geoaccumulation Index
IMHF	Indicador Hidromorfológico
IPS	Índice de Puluosensibilidad
IQ/FQ	Indicador Químico y Fisicoquímico
IR	Infrared
I_R	Ingestion Rate
IVG	In Vitro Gastro–intestinal method
IWRM	Integrated Water Resources Management
IWW	Industrial Wastewater
l.d.	Límite de Detección
m/m	Masa/Masa
m/v	Masa/Volumen
MBf	Multimetric Basque index Family
MIR	Mid–Infrared
MS&SR	Mass Balance & Soil Recapture
NADH	Nicotinamide Adenine Dinucleotide + Hydrogen
NBS	National Bureau of Standards
NCA	Norma de Calidad Ambiental
NCA-CMA	Norma de Calidad Ambiental–Concentración Máxima Admisible
NCA-MA	Norma de Calidad Ambiental–Media Anual
NiR	Nitrite Reductase
NMDS	Non–metric Multidimensional Scaling
NOB	Nitrite–Oxidizing Bacteria
NR	Nitrate Reductase

OMA	Objetivos Medioambientales
OUT	Operational Taxonomic Units
P	Precipitation/Precipitación
PBET	Physiologically Based Extraction Test
PC	Principal Component
PCA	Principal Component Analysis
PEL	Predicted Effects Level
PERI	Potential Ecological Risk Index
PERMANOVA	Permutational Analysis of Variance
PLI	Pollution Load Index
POC	Particulate Organic Carbon
PVPP	Polyvinylpolypyrrolidone
Q	Discharge/Caudal volumétrico
QI	Quality Index
qPCR	Quantitative Polymerase Chain Reaction
r.p.m	Revolution per Minute/Revoluciones por Minuto
RBALP	Relative Bioaccessibility Leaching Procedure
RfD_o	Oral Reference Dose
RIVM	Rijksinstituut voor Volksgezondheid en Milieu–Netherlands National Institute for Public Health and the Environment
rRNA	Ribosomal Ribonoclueic Acid
RT	Response Time
SBET	Simplified Bioaccessibility Extraction Test
SBS	Surface Bottom Sediment
SD	Standard Deviation
SE	Standard Error
SEM–EDS	Scanning Electron Microscopy with Energy Dispersive Spectroscopy
SHIME	Simulator of the Human Intestinal Microbial Ecosystem
SPE	Sustancias Poliméricas Extracelulares
SPM	Suspended Particulate Matter
Spp.	Species
SQG	Sediment Quality Guidelines
SS	Sólidos Suspendidos
T_A	Average Time
TC	Total Carbon
TCLP	Toxicity Characteristic Procedure
TEL	Threshold Effects Level
TIC	Total Inorganic Carbon
TMO	TNO (Nederlandse Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek–

	Netherlands Organisation for Applied Scientific Research) Gastrointestinal Model
TN	Total Nitrogen
TOC	Total Organic Carbon
TON	Total Organic Nitrogen
TS	Total Sulphur
UBM	Unified BARGE (Bioaccessibility Research Group of Europe) Bioaccessibility Method
UH	Unidad Hidrológica
UWW	Urban Wastewater
VBQ	Very Bad Quality
VGQ	Very Good Quality
W/V	Weight/Volume
W_{AB}	Body weight
WFD	Water Framework Directive
WWTP	Wastewater Treatment Plant
XRD	X–ray Diffraction

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Introducción

"Thousands have lived without love,
not one without water"

Wystan Hugh Auden (1907-1973)

1. Introducción

1.1. Evaluación del estado ecológico de los ríos: normativa europea, estatal y autonómica

Las aguas de la Comunidad Europea están sometidas a la creciente presión que supone el continuo crecimiento de la demanda de agua de buena calidad en cantidades suficientes para todos los usos. En este contexto, la Directiva 2000/60/CE del Parlamento Europeo y del Consejo de 23 de octubre de 2000, comúnmente denominada Directiva Marco del Agua (DMA), nació como respuesta a la necesidad de establecer un marco comunitario de actuación en el ámbito de la política en la Unión Europea, para la ordenación y regulación de la gestión —protección, regeneración y garantía de un uso sostenible— de los recursos hídricos, así como el establecimiento de objetivos medioambientales que asegurasen alcanzar una buena calidad ecológica de los ecosistemas acuáticos en un determinado plazo. Este sistema de gestión de las masas de agua está basado en la ordenación del territorio terrestre y marino en demarcaciones hidrográficas, compuestas a su vez, por una o varias cuencas hidrográficas y las aguas de transición, subterráneas y costeras asociadas a dichas cuencas. El término cuenca hidrográfica se define, tal y como establece el artículo 2, como "*la superficie de terreno*

cuya escorrentía superficial fluye en su totalidad a través de una serie de corrientes, ríos y, eventualmente, lagos hacia el mar por una única desembocadura, estuario o delta".

Conforme a la DMA, las autoridades competentes de cada Estado miembro elaboraron planes hidrológicos correspondientes a cada demarcación o cuenca hidrográfica. En ellos se recogen las medidas necesarias para la consecución de los objetivos medioambientales en los plazos temporales previstos, diseñadas de conformidad con los resultados derivados del análisis de las características de la demarcación, el estudio de las repercusiones de la actividad humana en el estado de las aguas y el análisis económico del uso del agua. De un modo general, en el artículo 4 se plantea la consecución de los siguientes objetivos medioambientales para las aguas superficiales, que eran de obligado cumplimiento en el año 2015, salvo cuando éstas incurrían en determinadas situaciones de excepción:

i) Prevenir el deterioro del estado de todas las masas de agua superficial.

ii) Proteger, mejorar y regenerar todas las masas de agua superficial con objeto de alcanzar un buen estado.

iii) Proteger y mejorar todas las masas de agua artificiales y muy modificadas con objeto de lograr un buen potencial ecológico y un buen estado químico de las aguas superficiales.

iv) Reducir progresivamente la contaminación procedente de sustancias prioritarias e interrumpir o suprimir gradualmente los vertidos, las emisiones y las pérdidas de sustancias peligrosas prioritarias.

Para la evaluación del cumplimiento de estos objetivos, los planes hidrológicos establecen además programas de seguimiento del estado de las aguas en cada demarcación hidrográfica. Con respecto a las aguas superficiales, estos programas incluyen el seguimiento de:

1) El volumen y el nivel de flujo en la medida en que sea pertinente para el estado ecológico y químico y el potencial ecológico.

2) El estado ecológico y químico y el potencial ecológico.

La incorporación de la DMA al ordenamiento jurídico español se realizó mediante la Ley 62/2003 de la Jefatura del Estado de 30 de diciembre, de medidas fiscales, administrativas y de orden social que incluye, en su artículo 129, la modificación del texto refundido de la Ley de Aguas, aprobado por el Real Decreto Legislativo 1/2001 del Ministerio de Medio Ambiente de 20 de julio.

Desde su publicación, tanto la normativa europea como la estatal han sufrido sucesivas modificaciones. Concretamente, la Directiva 2013/39/UE, del Parlamento Europeo y del Consejo de 12 de agosto de 2013, incluye modificaciones de las Directivas 2000/60/CE y 2008/105/CE relativas a las sustancias prioritarias en el ámbito de la política de aguas. Su implantación en el ordenamiento jurídico español se realizó a través del Real Decreto 817/2015 del Ministerio de Agricultura, Alimentación y Medio Ambiente de 11 de septiembre, por el que se establecen los criterios de seguimiento y evaluación del estado de las aguas superficiales y las normas de calidad ambiental en aplicación a lo dispuesto en la DMA. En su artículo 3 define:

i) El estado de las aguas superficiales como " *la expresión general del estado de una masa de agua superficial determinado por el peor valor de su estado ecológico y de su estado químico*".

ii) El estado ecológico como "*una expresión de la calidad de la estructura y funcionamiento de los ecosistemas acuáticos asociados a las aguas superficiales clasificados con arreglo a este real decreto*".

iii) El estado químico como "*una expresión de la calidad de las aguas superficiales que refleja el grado de cumplimiento de las normas de calidad ambiental (NCA) de las sustancias prioritarias y otros contaminantes del anexo IV de este real decreto".*

iv) El potencial ecológico como "*una expresión de la calidad de la estructura y el funcionamiento de los ecosistemas acuáticos asociados a una masa de agua artificial o muy modificada*".

Según dispone el Real Decreto 817/2015, la etapa fundamental de los programas de seguimiento de las aguas resulta ser el establecimiento previo de las denominadas condiciones

de referencia para cada tipo de masa de agua, dado que el estado o potencial ecológico debe calcularse como desviación con respecto a las características naturales o valores de los indicadores de calidad asociados a condiciones inalteradas. Para ello, las masas de agua superficial de cada demarcación hidrográfica deben clasificarse inicialmente en la categoría de ríos, lagos, aguas de transición o aguas costeras. Además, se catalogan como naturales, artificiales o muy modificadas según su naturaleza. Finalmente, para cada categoría de agua superficial, las masas de agua se clasifican por tipos utilizando el sistema A o el sistema B descritos en la sección 1.2 del anexo II de la DMA.

Posteriormente, el estado o potencial ecológico de las masas de agua de la categoría ríos debe evaluarse por la combinación de los siguientes indicadores, establecidos en el artículo 10 del Real Decreto 817/2015:

1) Indicadores biológicos:
    1.1) Composición y abundancia de la flora acuática.
    1.2) Composición y abundancia de la fauna bentónica de invertebrados.
    1.3) Composición, abundancia y estructura de la fauna ictiológica.
2) Indicadores hidromorfológicos que afectan a los indicadores biológicos:
    2.1) Régimen hidrológico:
        2.1.1) Caudales e hidrodinámica del flujo de las aguas.
        2.1.2) Conexión con masas de agua subterránea.
    2.2) Continuidad del río.
    2.3) Condiciones morfológicas:
        2.3.1) Variación de la profundidad y anchura del río.
        2.3.2) Estructura y sustrato del lecho del río.
        2.3.3) Estructura de la zona ribereña.
3) Indicadores químicos y fisicoquímicos que afectan a los indicadores biológicos:

3.1) Generales:

3.1.1) Condiciones térmicas.

3.1.2) Condiciones de oxigenación.

3.1.3) Salinidad.

3.1.4) Estado de acidificación.

3.1.5) Condiciones en cuanto a nutrientes.

3.2) Contaminantes específicos vertidos en cantidades significativas.

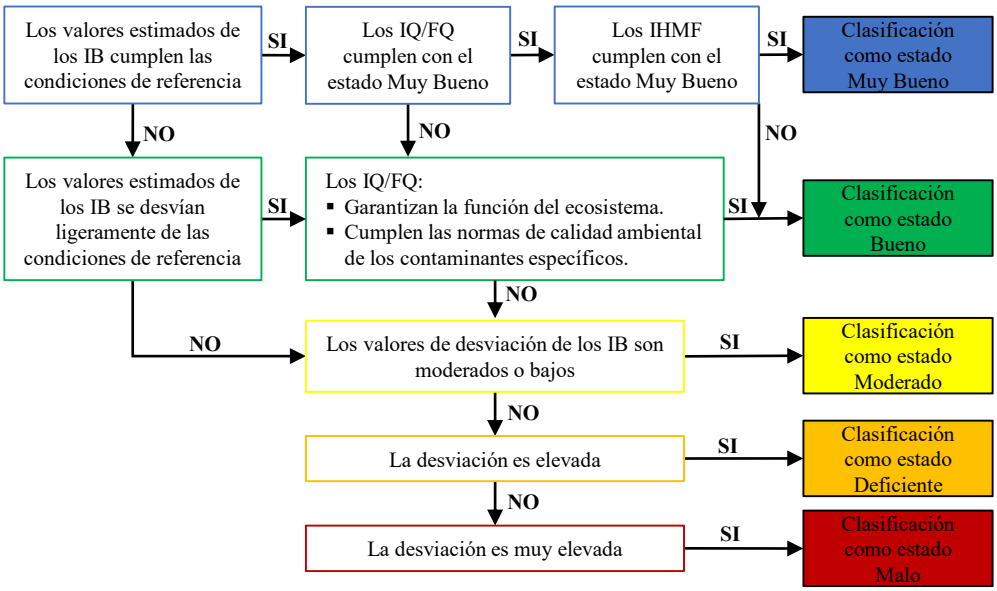

Fig. 1.1. *Esquema del procedimiento iterativo para la calificación del estado o potencial ecológico mediante la evaluación de los indicadores biológicos (IB), los químicos y fisicoquímicos (IQ/FQ), y los hidromorfológicos (IHMF). Fuente: Figura modificada del Real Decreto 817/2015*

Esta evaluación se realiza a través de un proceso iterativo (Fig. 1.1), que comprende el análisis de los indicadores biológicos, seguido del análisis de los indicadores químicos y fisicoquímicos y, finalmente, el análisis de los indicadores hidromorfológicos. Para la calificación del estado o potencial ecológico, se establecen 5 clases de estado (Muy Bueno, Bueno, Moderado, Deficiente y Malo), que vendrán determinadas por el elemento de calidad

cuyo resultado final sea el más desfavorable. Por tanto, la calificación del estado o potencial ecológico se corresponde, en primer lugar, con la peor de las valoraciones efectuadas para cada uno de los indicadores biológicos. Cuando esta calificación resulta Buena o Muy Buena, se completa con la valoración de los indicadores químicos y fisicoquímicos generales que afectan a los indicadores biológicos. La valoración de los indicadores hidromorfológicos únicamente se requiere para discernir entre la calificación de Buen Estado y Muy Buen Estado.

En el ámbito de competencia de la Comunidad Autónoma del País Vasco, el ente público Agencia Vasca del Agua – Ur Agentzia (URA), creado por la Ley de Aguas 1/2006 del Parlamento Vasco de 23 de junio y adscrito al Departamento de Medio Ambiente y Ordenación del Territorio del Gobierno Vasco, tiene establecida una red de seguimiento del estado ecológico de los ríos pertenecientes a la demarcación hidrográfica del Cantábrico Oriental, en conformidad con lo exigido tanto por la legislación europea como por la estatal. Esta red de seguimiento tiene por objeto la evaluación, por un lado, del estado biológico y, por el otro, del estado químico de los ríos.

Tabla 1.1. *Relación de índices para la evaluación de los indicadores biológicos. Fuente: Elaboración propia a partir del informe de la Agencia Vasca del Agua – Ur Agentzia (URA, 2019a)*

Indicador	Índice
Composición y abundancia de la flora acuática	
❑ Organismos fitobentónicos	Índice de Puluosensibilidad (IPS)
❑ Macrófitos	Índice Biológico de Macrófitos en Ríos (IBMR)[a]
Composición y abundancia de la fauna bentónica de invertebrados	Índice Multimétrico Vasco a nivel familia (en inglés, Multimetric Basque index Family (MBf))
Composición, abundancia y estructura de la fauna ictiológica	Índice CFI (en inglés, Cantabrian Fish Index)[b]

(a) En fase de recopilación de datos para su aplicación.
(b) Tiene un menor peso en la determinación de la calidad biológica dada su reciente implantación y ausencia de intercalibración.

La red de seguimiento del estado biológico de los ríos se centra en la determinación de los índices relativos a cada indicador biológico (Tabla 1.1) y posterior cálculo del denominado Ratio de Calidad Ecológica (en inglés, Ecological Quality Ratio (EQR)) que debe oscilar entre 0 y 1; es decir, el cociente entre los valores observados de los índices y los correspondientes

a las condiciones de referencia para cada tipología de masa de agua (URA, 2019a). La consecución de un buen estado ecológico implica, por tanto, el cumplimiento del valor del EQR (límite entre Bueno y Moderado) para cada indicador biológico.

Por otro lado, la explotación de la red de seguimiento del estado químico de los ríos permite completar la evaluación del estado ecológico mediante la valoración de los indicadores químicos y fisicoquímicos que afectan a los indicadores biológicos, determinando los parámetros individuales relativos a las condiciones fisicoquímicas generales junto con un índice integrador (Índice Fisicoquímico Referenciado (IFQ-R)), así como las sustancias preferentes contempladas en el anexo V del Real Decreto 817/2015. Asimismo, esta red proporciona información para evaluar el estado químico relativo a las sustancias prioritarias y otros contaminantes contemplados en el anexo IV del Real Decreto 817/2015.

La Tabla 1.2 resume los parámetros individuales relativos a condiciones fisicoquímicas generales, y sus límites de cambio de estado en función de la correspondiente tipología de masa de agua, tal y como indica en su Anexo II el Real Decreto 817/2015. Para el caso de las variables DBO_5 (demanda biológica de oxígeno) y DQO (demanda química de oxígeno), la Agencia Vasa del Agua – Ur Agentzia (URA, 2019b) tiene en cuenta las indicaciones que marca el Apéndice 8 de la Normativa del Plan Hidrológico de la Demarcación Hidrográfica Cantábrico Oriental (2015-2021), en el que se especifican los valores de referencia en el Dominio Público Hidráulico para el cumplimiento de los objetivos medioambientales aguas abajo de los vertidos. Mientras que las variables pH y porcentaje de oxígeno se valoran utilizando como estadístico el valor promedio anual, las variables restantes se evalúan mediante el percentil 75 anual (URA, 2019b). Conjuntamente, la Agencia Vasca del Agua – Ur Agentzia calcula, como herramienta complementaria, el índice IFQ-R (Eq. 1.1) a partir de las siguientes variables fisicoquímicas capaces de reflejar la influencia de la actividad humana: condiciones de oxigenación (porcentaje de saturación de oxígeno ($\%O_2$), DQO y DBO_5) y condiciones relativas a nutrientes (fósforo total (PT), amonio (NH_4), nitrito (NO_2) y nitrógeno total (NT)) (URA, 2019b).

$$\begin{aligned} IFQ - R = {} & 0{,}35783460 + 0{,}00231993\ (\%O_2) - 0{,}0878411\ \mathrm{Log}_{10}(NH_4) \\ & - 0{,}12033473\ \mathrm{Log}_{10}(DBO_5) - 0{,}10490488\ \mathrm{Log}_{10}(DQO) - 0{,}06871787\ \mathrm{Log}_{10}(NO_2) \\ & - 0{,}07353095\ \mathrm{Log}_{10}(PT) - 0{,}10340487\ \mathrm{Log}_{10}(NT) \end{aligned} \quad (1.1)$$

Tabla 1.2. *Relación de parámetros individuales relativos a las condiciones fisicoquímicas generales y sus límites de cambio de estado (Muy Bueno (MB), Bueno (B) y Moderado (Mo)) correspondientes a cada tipología de masa de agua. $\%O_2$: porcentaje de saturación de oxígeno; DBO_5: demanda biológica de oxígeno; DQO: demanda química de oxígeno. Fuente: Tabla modificada del informe de la Agencia Vasca del Agua – Ur Agentzia (URA, 2019b)*

Indicador	Unidad	Tipologías	Límites de cambio de clase de estado	
			MB/B	B/Mo
pH	–	R-T09, R-T12, R-T15, R-T22, R-T23, R-T26, R-T29 y R-T32	6,5 – 8,7	6 - 9
		R-T30	6 – 8,4	5,5 - 9
% O_2	%	R-T09, R-T12, R-T15, R-T22, R-T26, R-T29 y R-T32	70 -100	60 – 120
		R-T23	90 – 105	70 – 120
		R-T30	70 - 105	60 - 120
NH_4^+	mg NH_4^+ L^{-1}	R-T09, R-T12, R-T15, R-T22, R-T23, R-T26, R-T30 y R-T32	0,2	0,6
		R-T29	0,3	1
NO_3^-	mg NO_3^- L^{-1}	R-T09, R-T12, R-T15, R-T22, R-T26, R-T29, R-T30 y R-T32	10	25
		R-T23	8	15
PO_4^{3-}	mg PO_4^{3-} L^{-1}	R-T09, R-T12, R-T22, R-T23, R-T26, R-T29, R-T30 y R-T32	0,2	0,4
		R-T15	0,4	0,5
DBO_5	mg L^{-1}	R-T09, R-T12, R-T15, R-T22, R-T23, R-T26, R-T29, R-T30 y R-T32	2	5
DQO	mg L^{-1}	R-T09, R-T12, R-T15, R-T22, R-T23, R-T26, R-T29, R-T30 y R-T32	9,9	17,0

La clase de estado relativa al índice IFQ-R se determina comparando el valor del percentil 25 de la serie de resultados anuales con los límites de cambio de clase de estado (Muy Bueno/Bueno: 0,646; Bueno/Moderado: 0,513; Moderado/Deficiente: 0,381; Deficiente/Malo: 0,249) establecidos en el Anexo VIII del Plan Hidrológico de la Demarcación Hidrográfica Cantábrico Oriental (2015-2021). Los objetivos medioambientales relativos al índice IFQ-R en ríos se cumplen, por tanto, cuando el 75% de las muestras recogidas durante un año están en las clases Bueno o Muy Bueno. En ningún caso el valor de una muestra puntual puede ser

inferior al umbral Moderado/Deficiente, aunque el resto de las muestras mensuales hayan obtenido calificaciones de clase de estado Bueno o Muy Bueno (URA, 2019b).

Finalmente, el estado relativo a las condiciones fisicoquímicas generales se evalúa a partir de los resultados obtenidos para el IFQ-R y los parámetros individuales, de acuerdo con la Tabla 1.3.

Tabla 1.3. *Matriz de evaluación del estado relativo a las condiciones fisicoquímicas generales. Fuente: Tabla modificada del informe de la Agencia Vasca del Agua – Ur Agentzia (URA, 2019b)*

Estado relativo al IFQ-R	**Estado relativo a los indicadores fisicoquímicos individuales**		
	Muy Bueno	Bueno	Moderado
Muy Bueno	Muy Bueno	Bueno	Moderado
Bueno	Bueno	Bueno	Moderado
Moderado	Moderado	Moderado	Moderado
Deficiente	Deficiente	Deficiente	Deficiente
Malo	Malo	Malo	Malo

La evaluación del estado fisicoquímico relativo a las sustancias preferentes se realiza mediante el análisis del cumplimiento de las normas de calidad ambiental (NCA) indicadas en el anexo V del Real Decreto 817/2015. Los criterios para la evaluación son los siguientes (URA 2019b):

i) Se considera un estado de clase Muy Bueno cuando la media aritmética anual para todas las sustancias analizadas se encuentra por debajo del 50% de la NCA-MA (media anual) y no hay ningún valor puntual que la supere, o todos los resultados son menores que el límite de cuantificación.

ii) Se considera un estado de clase Bueno cuando la media aritmética anual para todas las sustancias analizadas en el punto de control es inferior o igual a la NCA-MA.

iii) Se considera que el estado no alcanza la clase Bueno cuando la media aritmética anual de alguna de las sustancias analizadas en el punto de control supera la NCA-MA.

Tras la determinación del estado ecológico, la evaluación del estado de los ríos se completa con la clasificación del estado químico de las masas de agua, mediante el análisis de conformidad de la concentración de las sustancias prioritarias y otros contaminantes con las NCA recogidas en el anexo IV del Real Decreto 817/2015. Los criterios para la evaluación son los siguientes (URA 2019b):

i) Se considera un estado de clase Bueno cuando los valores medios anuales son inferiores o iguales a la NCA-MA (media anual) y no hay ningún valor puntual que sobrepase la NCA-CMA (concentración máxima admisible).

ii) Se considera que el estado no alcanza la clase Bueno cuando la media aritmética de las concentraciones de un contaminante en un punto de control supera el valor NCA-MA o si un valor puntual de un contaminante supera la NCA-MA.

1.2. Aplicabilidad del sedimento en los programas de monitoreo del estado ecológico

La DMA, así como otras normativas desarrolladas en el ámbito de la política de aguas, consideran el Agua un eje esencial en la gestión integral de las cuencas hidrográficas. Sin embargo, lejos de ser un elemento aislado, forma parte de un sistema de compartimentos del medio acuático, interconectados, al que también pertenecen las matrices Biota y Sedimento. Mientras que la Biota y, en especial, el Agua son matrices que cuentan con un amplio desarrollo normativo a nivel europeo, los Sedimentos carecen de un marco legislativo sólido que enfatice y promueva su potencial para la evaluación del estado ecológico de las masas de agua.

Numerosos autores han evidenciado las deficiencias que la DMA tiene en lo que a los sedimentos se refiere. Entre otros, Borja et al. (2004) señalaron lo significativo que es el hecho de que el término “agua” se cite en 373 ocasiones a lo largo de toda la Directiva, mientras que los conceptos de “sedimento” o “biota” sólo se mencionan explícitamente 7 y 4 veces, respectivamente. Asimismo, Förstner (2002) advirtió que obviar el papel de los sedimentos como fuente secundaria de contaminantes a largo plazo puede conducir a un análisis poco fiable del riesgo de alcanzar un buen estado de las masas de agua.

Como resultado de la propuesta de la Comisión Europea (COM(2011) 876 final), la entrada en vigor de la Directiva 2013/39/UE, del Parlamento Europeo y del Consejo de 12 de agosto de 2013, que incluye modificaciones de las Directivas 2000/60/CE y 2008/105/CE relativas a las sustancias prioritarias en el ámbito de la política de aguas, supuso una leve evolución de la normativa. En esta se define por primera vez el término matriz como "*compartimento medioambiental, que puede ser el agua, los sedimentos o la biota*", y al que se aplican las normas de calidad ambiental (NCA) para el seguimiento de las concentraciones de sustancias prioritarias y peligrosas. Sin embargo, para cada sustancia de la lista se especifica una matriz de seguimiento por defecto, de manera que sigue considerándose prioritaria el Agua, en ocasiones la Biota (peces), y como opción para los Estados miembro el Sedimento (Art. 3.3).

Ciertamente, el artículo 20 del Real Decreto 817/2015 para la aplicación de las NCA en sedimento y biota, señala que para las sustancias prioritarias indicadas con los números 5, 15, 16, 17, 21, 28, 34, 35, 37, 43 y 44 en el Anexo IV A, se aplicarán las NCA de la biota establecidas en el citado anexo. Para el resto de las sustancias se aplicarán las NCA del agua establecidas en el anexo IV A. Por su parte, para las sustancias preferentes y contaminantes específicos indica que los órganos competentes podrán aplicar las NCA a los sedimentos y la biota en relación con las sustancias preferentes enumeradas en el Anexo V A, si ofrecen al menos el mismo grado de protección que las NCA establecidas. Estas NCA se establecerán con arreglo al procedimiento fijado en el Anexo VII y deberán proporcionar el mismo nivel de protección en toda la demarcación hidrográfica (URA, 2019b).

Como reclamo de la aplicabilidad del sedimento en la evaluación del estado de las masas de agua, Förstner y Salomons (2010) plantearon que el desarrollo de un Modelo Conceptual de una Cuenca Hidrográfica (en inglés, Conceptual River Basin Model) debe incluir la identificación de las fuentes de sedimento, las rutas tanto de los propios sedimentos como de los contaminantes asociados dentro y entre los diferentes compartimentos medioambientales, así como el rol de las zonas de acumulación (Owens, 2005). Sin embargo, a tenor de los múltiples elementos que pueden llegar a influir en la calidad y cantidad de sedimento (Fig. 1.2), su introducción en la normativa puede llegar a ser una labor desafiante. No sólo conllevaría la elaboración de estudios integrados sobre procesos hidromecánicos,

biológicos y geoquímicos, sino también evaluaciones de riesgo y desarrollo de herramientas de decisión para la realización de medidas técnicas y sostenibles, a escala de cuenca, que consideren los diferentes aspectos de los sedimentos (Förstner, 2002).

En los últimos años, el Sedimento ha adquirido un auge científico importante, como lo atestiguan revistas especializadas en este dominio, entre otras, *Journal of Soils and Sediments*. En una de sus editoriales (Owens y Xu, 2011), realizaba una amplia revisión de los últimos avances en la investigación, identificando las principales cuestiones, varias de las cuales se abordan a continuación.

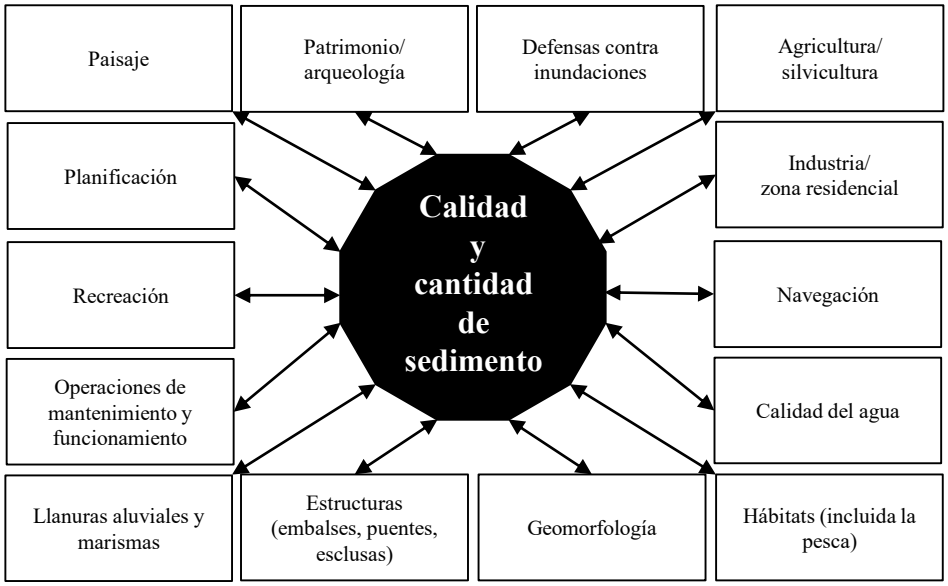

Fig. 1.2. *Principales elementos que impactan sobre la calidad y cantidad de sedimento en una cuenca hidrográgica. Fuente: Figura modificada de Owens, 2005*

1.2.1. Calidad del sedimento y evaluación del impacto

Las cuencas urbanas con abundantes fuentes de contaminación han sufrido fuertes impactos sobre su estado ecológico (Taylor y Owens, 2009), incidiendo no solo sobre la salud de los ecosistemas sino también en la del ser humano (Horowitz y Stephens, 2008).

Los contaminantes entran en el sistema acuático y los sedimentos actúan como sumideros potenciales (Violintzis et al., 2009). La toxicidad de estos contaminantes viene

determinada, no sólo por su contenido total en el sedimento, sino también por la forma química en la que se encuentren y, por ende, por su biodisponibilidad (Pagnanelli et al., 2004; Clozel et al., 2006; Zhang et al., 2014). Tal y como recoge la Fig. 1.3, las especies de origen antropogénico emitidas al medio acuático pueden perturbar su equilibrio y comportamiento natural, lo que conlleva una alteración de su especiación medioambiental (forma química) y su disponibilidad biológica. En consecuencia, su toxicidad también se ve afectada ya que depende del comportamiento y posterior destino de las especies en los organismos, es decir, su adsorción por las membranas celulares, su transporte e incorporación a las estructuras o enzimas, así como su enriquecimiento y excreción del organismo (Michalke, 2003).

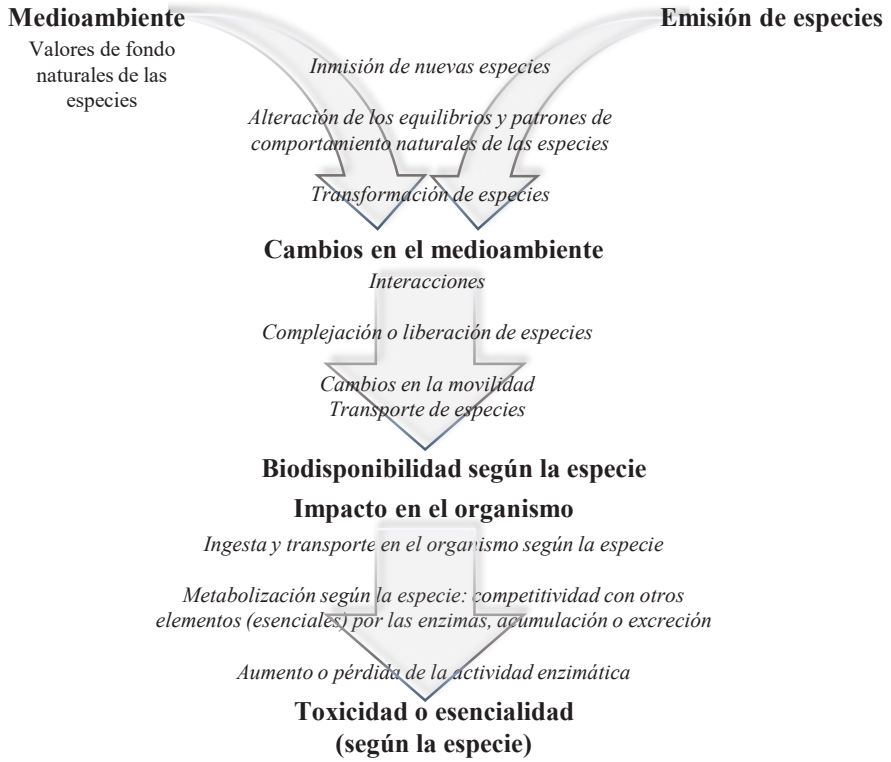

Fig. 1.3. *Relación de dependencia entre la especiación medioambiental, la biodisponibilidad y la toxicidad de las especies. Fuente: Figura modificada de Michalke, 2003*

Es por esto por lo que la relación de la calidad de los sedimentos y la salud tanto ecosistémica como humana es de creciente interés, tal y como lo atestigua el rápido aumento de publicaciones científicas en la última década (Liu et al., 2009; Yi et al., 2011; Ccanccapa

et al., 2016; Enuneku et al., 2018). Sin embargo, si bien existe un número considerable de investigaciones centradas en el impacto del sedimento en los ecosistemas acuáticos (principalmente, en organismos como invertebrados y peces), los trabajos sobre el impacto en la salud humana son limitados (Owens y Xu, 2011).

1.2.2. Procesos físicos y biogeoquímicos

El estudio de los procesos físicos, químicos y biogeoquímicos que ocurren en la interfase Agua-Sedimento es una de las áreas de investigación clave ya que evalúa tanto el grado de movilización de contaminantes (especiación medioambiental) en el sedimento como su intercambio entre ambas matrices (Owens and Zhu, 2011). Por esta razón, el sedimento no sólo se considera sumidero sino también fuente de contaminantes en el medio acuático (Salomons and Brill, 1987). De hecho, los sedimentos están constituidos por múltiples fases (materia orgánica, óxidos, sulfuros, carbonatos y minerales de arcilla o limo) cuya abundancia relativa depende del pH, las condiciones redox, el régimen hidrológico y las áreas de deposición (Burton et al., 2006; Zhang et al., 2014). En el caso de los metales, su movilidad queda definida por numerosos procesos como la adsorción/desorción, precipitación/disolución y complejación/decomplejación que tienen lugar en la interfase Agua-Sedimento. Como ejemplo, mientras que una elevada capacidad de intercambio catiónico —favorecida por una mayor presencia de materia orgánica, minerales de arcilla, y óxidos de Fe y Al en el sedimento— reduce la movilidad de los metales, la salinidad y la presencia de componentes orgánicos solubles en el agua intersticial pueden promover su liberación (Du Laing et al., 2009).

Asimismo, estudios recientes han demostrado la implicación de los microorganismos y plantas en la movilización de contaminantes en el sedimento. Entre otros, García-Aragón et al. (2011) demostraron que las sustancias poliméricas extracelulares (SPE) secretadas por los microorganismos reducen la erosión y resuspensión del sedimento, limitando así la liberación de contaminantes al agua; por el contrario, los exudados o sustancias secretadas por las plantas favorecen la remobilización de metales (Xu y Jaffe, 2006).

1.2.3. Dinámica de los sedimentos en la cuenca

A lo largo de las últimas décadas, la modelización de la erosión y transporte de los sedimentos ha sido el eje principal de una gran variedad de estudios (Hajigholizadeh et al., 2018). Sin embargo, la constatación de que el sedimento es un importante vector de transporte de contaminantes y nutrientes asociados (Förstner y Owens, 2007), ha suscitado un creciente y renovado interés por determinar su origen y rutas de transporte a través de los medios acuáticos (Owens y Xu, 2011).

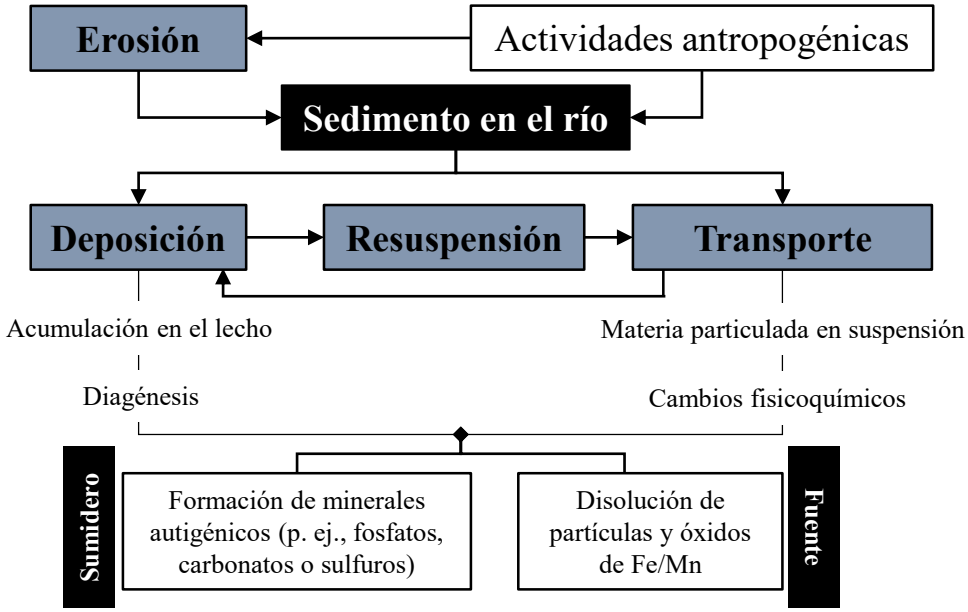

Fig. 1.4. *Esquema de interdependencia entre la dinámica de sedimentos (erosión, transporte, deposición y resuspensión) y su función como vector de transporte de la contaminación a lo largo de una cuenca fluvial. Fuente: Figura modificada de Taylor y Owens, 2009*

Numerosos autores han constatado la implicación de los eventos lluviosos en la activación del transporte de sedimentos y contaminación asociada (Rodríguez-Blanco et al., 2010; Cerro, 2014; Martínez-Santos et al., 2014). Además, dada la participación de la materia particulada en suspensión en el devenir de los contaminantes (Fig. 1.4), la distribución del tamaño de partícula y la influencia que el régimen hidrológico tiene sobre esta, han sido el eje principal de diversas investigaciones (Palleiro et al., 2013; Matsunaga et al., 2014; Le Meur et al., 2016). Ciertamente, sus propiedades físicas —distribución del tamaño de partícula, forma,

porosidad y densidad, entre otras— influyen significativamente en la dinámica del sedimento (Mladenović et al., 2015) y, por tanto, en el transporte de contaminantes a lo largo de la cuenca fluvial. Y es que las partículas más pequeñas poseen unas características fisicoquímicas determinadas (p. ej., una elevada área superficial o mayor contenido de minerales arcillosos, óxidos e hidróxidos de Fe y Mn, carbonatos y materia orgánica) que les confieren una alta capacidad de intercambio catiónico (Hardy y Cornu, 2006; Parizanganeh, 2008; Guven y Akinci, 2013) y, en consecuencia, de acumulación de contaminantes.

1.2.4. Interacción sedimento-ecología

La relación entre la calidad del sedimento y la salud ecosistémica es bidireccional. Si bien la primera influye en el funcionamiento de los organismos acuáticos, estos, a su vez, contribuyen a la modificación de las características fisicoquímicas y biológicas de los primeros.

Como se ha comentado anteriormente, la vegetación y los organismos acuáticos (p. ej., peces, macroinvertebrados o microorganismos) juegan un papel fundamental en la erosión y estabilización (deposición/resuspensión) del sedimento (De Baets et al., 2011; De Vries, 2011; García-Aragón et al., 2011). Además, la enorme biodiversidad y versatilidad metabólica de las comunidades microbianas presentes en el sedimento les confiere un papel clave en los procesos ecológicos fluviales. Las bacterias, junto con los hongos, llevan a cabo la función esencial de la descomposición de la materia orgánica, así como también participan en muchos procesos implicados en los ciclos biogeoquímicos de los principales elementos (C, N, P, S, etc.) (Pankhurst et al. 1997). Sin embargo, la densidad y biodiversidad microbiana depende de numerosos factores fisicoquímicos y biológicos (presencia de contaminantes como metales, disponibilidad de materia orgánica, flujo de nutrientes, concentración de oxígeno, temperatura, pH, tamaño de partícula, interacción de especies invasoras, entre otros) (Ricciardi et al., 2009; Bouskill et al., 2010). En consecuencia, la sostenibilidad funcional de los procesos ecológicos antes mencionados puede verse comprometida por la calidad del sedimento, ya que la excepcional biodiversidad microbiana, tanto estructural como funcional, aporta *a priori* una mayor estabilidad (resistencia y resiliencia) frente a perturbaciones (naturales y/o antropogénicas) en el ecosistema (Singh, 2015; Bender et al., 2016).

1.3. Indicadores de la calidad del sedimento

La proliferación del estudio de los sedimentos en las diferentes áreas de investigación abordadas en el anterior apartado demuestra su posible aplicabilidad en los programas de monitoreo del estado ecológico de una cuenca. Con esta finalidad, se describen a continuación varios indicadores de calidad que pueden ser de gran ayuda en el establecimiento de estos programas.

1.3.1. Indicadores biológicos: abundancia, biodiversidad y actividad de la comunidad microbiana

La fuerte urbanización e industrialización de las últimas décadas ha derivado en un aumento de las fuentes antropogénicas puntuales y difusas (p.ej. cultivos de legumbres, uso de fertilizantes para la agricultura, quema de combustibles fósiles (Gruber et al., 2008)), que junto con los vertidos de aguas residuales tratadas y no tratadas (Ali et al., 2012; Metcalf y Eddy, 2014), han incrementado la biodisponibilidad de los compuestos inorgánicos nitrogenados (especialmente nitratos) en los ríos (Seitzinger et al., 2008; Graham et al., 2010; Decleyre et al., 2015). Como resultado, el balance global del nitrógeno ha sufrido un marcado desequilibrio, dando lugar a diversos problemas ambientales, entre los que destacan la eutrofización de los sistemas acuáticos (Mcdowell et al., 2010), la pérdida de biodiversidad y la reducción de la resiliencia ecosistémica (Bai et al., 2010).

En esta coyuntura, la enorme biodiversidad y versatilidad metabólica de las comunidades bacterianas presentes en el sedimento, y su rápida respuesta ante cualquier perturbación en el ecosistema, les confiere un papel clave en la restauración de los sistemas acuáticos y evaluación de su estado ecológico (Chaer et al., 2009; Martins et al., 2011; Guo et al, 2012). Numerosos trabajos basados en el estudio de la diversidad bacteriana en sedimentos de lagos de agua dulce han revelado que *Proteobacteria, Chloroflexi, Actinobacteria, Bacteroidetes* y *Planctomycetes* son los filos más predominantes. Muchas bacterias involucradas en el ciclo del nitrógeno pertenecen a estos filos: las bacterias oxidantes del amonio (en inglés, ammonia-oxidizing bacteria (AOB)) del género *Betaproteobacteria*, las bacterias oxidantes del nitrito (en inglés, nitrite-oxidizing bacteria (NOB)) del género *Nitrobacter* (también

pertenecientes al género *Nitrospira* del filo *Nitrospira*), las bacterias desnitrificantes (en inglés, denitrifying bacteria (DNB)) o las bacterias anaerobias oxidantes del amonio (en inglés, anaerobic ammonia-oxidizing bacteria (AnAOB)) del orden *Planctomycetales* (Martins et al., 2011). Sin embargo, poco se sabe sobre la abundancia de las comunidades microbianas en los sedimentos. Recientemente, ha comenzado a aplicarse la técnica de la reacción en cadena de la polimerasa cuantitativa en tiempo real (en inglés, real-time quantitative polymerase chain reaction (qPCR)) para la cuantificación del número de copias de genes funcionales o filogenéticos específicos en el ADN de bacterias en sedimentos (Nakamura et al., 2006; Bedard et al., 2007; Bulow et al., 2008; Martins et al., 2011).

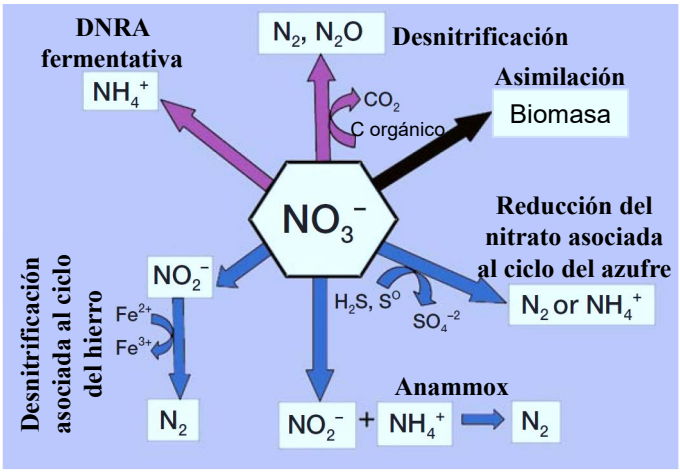

Fig. 1.5. *Diagrama conceptual de diferentes vías de eliminación de nitratos. DNRA: reducción desasimilatoria del nitrato a amonio (en inglés, dissimilatory nitrate reduction to ammonium). Flechas negras: proceso asimilativo; azules: proceso desasimilatorio autotrófico; y rosas: proceso desasimilatorio heterotrófico. Fuente: Figura modificada de Burgin y Hamilton, 2007*

Dentro del ciclo del nitrógeno, la desnitrificación en sedimentos ha sido objeto de estudio de cuantiosas investigaciones ya que constituye la única vía por la que este (N) retorna a la atmósfera (Arce et al., 2015), además de no depender de la presencia de compuestos nitrogenados generados en otros procesos (Burgin y Hamilton, 2007). Sin embargo, puede contribuir al aumento de la lluvia ácida, el smog fotoquímico y la pérdida de ozono estratosférico (efecto invernadero), como consecuencia de la emisión de NO y N_2O durante una desnitrificación incompleta (Gruber et al., 2008, Lu et al., 2014).

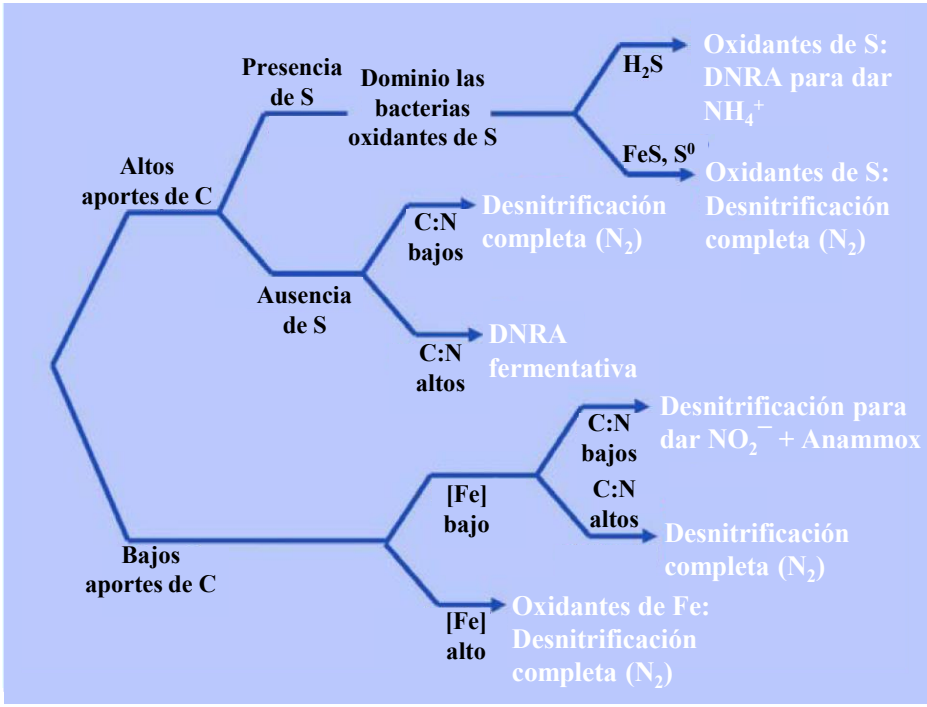

Fig. 1.6. *Predominio de las diferentes vías de eliminación de nitratos de la Fig. 1.5 en función de la disponibilidad de carbono orgánico lábil (C), el ratio entre el carbono orgánico y el nitrato lábil (C:N) y la presencia de hierro o azufre (como azufre elemental (S^0), sulfuros libres (H_2S o S^{2-}) o sulfuros metálicos como FeS). Fuente: Figura modificada de Burgin y Hamilton, 2007*

La desnitrificación es un proceso facultativo anaerobio que consta de cuatro etapas: (1) la reducción del nitrato (NO_3^-) a nitrito (NO_2^-) catalizada por la enzima Nitrato Reductasa (Nar), (2) la reducción de nitrito a óxido nítrico (NO) catalizada por la enzima Nitrito Reductasa (Nir), (3) la reducción del óxido nítrico a óxido nitroso (N_2O) catalizada por la enzima Óxido Nítrico Reductasa (Nor), y (4) la reducción del óxido nitroso a nitrógeno gas (N_2) catalizada por la enzima Óxido Nitroso Reductasa (Nos) (Knowles, 1982; Zumft, 1997; Moreno-Vivián et al., 1999). Puede llevarse a cabo tanto por organismos quimioorganoheterótrofos que utilizan la materia orgánica como donador de electrones, como por quimiolitoautótrofos que utilizan el NO_3^-, NO_2^-, NO y N_2O para oxidar compuestos inorgánicos. Sin embargo, hasta la fecha sólo se conocen unos pocos quimiolitoautótrofos capaces de desnitrificar (Sievert et al., 2008), mientras que los quimioorganoheterótrofos son más abundantes debido a su ubicuidad tanto en suelos como en hábitats acuáticos (Shapleigh, 2006; Verbaendert et al., 2011).

En consecuencia, es razonable considerar el estudio de la **abundancia** y la **biodiversidad** (estructural y funcional) de la **comunidad bacteriana en los sedimentos**, así como la **actividad** de cada una de las **enzimas** que catalizan las diferentes etapas de la desnitrificación como indicadores biológicos del estado ecológico de una cuenca, debido al profundo efecto que tienen no sólo sobre la tasa de desnitrificación sino también sobre su completación. En este aspecto, debe incidirse en el hecho de que no todas las bacterias desnitrificantes poseen todos los genes codificadores de las enzimas que catalizan la desnitrificación, desde NO_3^- hasta N_2. Mientras que los genes *nirS* y *nirK* que codifican la enzima Nitrito Reductasa están presentes en el genoma de todas las bacterias desnitrificantes (Azziz et al., 2017), se ha advertido una ausencia del gen *nosZ* codificador de la enzima Óxido Nitroso Reductasa (Liggi et al, 2014), lo que deriva en una desnitrificación incompleta y, en consecuencia, en la emisión de gases de efecto invernadero (N_2O). Además, durante años se consideró que la desnitrificación era el principal proceso de eliminación de nitratos en los sedimentos de los ríos (Burgin y Hamilton, 2007). Sin embargo, estudios recientes han revelado la existencia de otros procesos de reducción de nitratos (Fig. 1.5), que compiten con la desnitrificación y cuyo predominio depende en gran medida de factores tanto bióticos como abióticos (Fig. 1.6) (Burgin and Hamilton, 2007; Decleyre et al., 2015):

i) **Reducción desasimilatoria del nitrato a amonio** (en inglés, dissimilatory nitrate reduction to ammonium (**DNRA**)). Es un proceso facultativo anaerobio que consta de dos etapas: (1) la reducción del nitrato (NO_3^-) a nitrito (NO_2^-) catalizada por la enzima Nitrato Reductasa (Nap) y (2) la reducción de nitrito a amonio (NH_4^+) catalizada por la enzima Nitrito Reductasa (Nir o Nrf) (Moreno-Vivián et al., 1999; Decleyre et al., 2015). Puede llevarse a cabo tanto por organismos heterótrofos que utilizan la materia orgánica como donador de electrones (DNRA fermentativa), como por organismos quimiolitoautótrofos que utilizan el nitrato para oxidar sulfuros u otros compuestos de azufre (Burgin y Hamilton, 2007). Numerosos estudios han coincidido en el aumento de la DNRA en detrimento de la desnitrificación en condiciones de altas temperaturas, salinidad, aportes de carbono y reducción de sulfatos (Giblin et al., 2013).

ii) **Reducción de nitrato asociada al ciclo del hierro.** Puede efectuarse tanto por vías bióticas como abióticas, y verse favorecida en condiciones de bajas temperaturas

y aportes de nitrato, así como pHs cercanos a la neutralidad (Weber et a., 2006; Burgin y Hamilton, 2007).

iii) **Oxidación anaerobia del amonio** (en inglés, anaerobic ammonium oxidation (**Anammox**)) es un proceso quimiolitoautotófico en el que el amonio (NH_4^+) reacciona con el nitrito (NO_2^-) en condiciones anaerobias para formar nitrógeno gas (N_2). Poco se sabe aún sobre este proceso, aunque sí se han identificado bacterias pertenecientes al orden *Planctomycetes* involucradas en el proceso Anammox (Burgin y Hamilton, 2007; Martins et al., 2011). Conjuntamente, se ha observado que predomina en aguas anóxicas con escasez de carbono lábil o un exceso de nitrógeno en relación con la disponibilidad de carbono.

1.3.2. Indicadores fisicoquímicos

Evaluar la interacción entre los factores abióticos y bióticos del sedimento es fundamental para determinar el estado ecológico de una cuenca con relación a la calidad fisicoquímica del sedimento. Existen numerosos factores abióticos del sedimento que influyen sobre la composición, biodiversidad y actividad de la comunidad microbiana; entre ellos, destacan:

i) **Nutrientes y materia orgánica.** Las comunidades microbianas del sedimento participan en la descomposición de la materia orgánica, así como la transformación de los nutrientes presentes en el sedimento, influyendo consecuentemente en sus concentraciones. Por tanto, los productos del metabolismo de estas comunidades constituyen parámetros útiles para la medición de su actividad. Inversamente, la disponibilidad de materia orgánica y nutrientes repercute en la actividad y composición (estructural y funcional) de las comunidades microbianas (Torres e Inglett, 2010; Sinkko et al., 2013; Huang et al., 2017). Además, no sólo la cantidad sino la composición de la materia orgánica y la forma química de los nutrientes (p. ej., distintos compuestos de nitrógeno orgánico o inorgánico) tiene un profundo efecto sobre la actividad microbiana. Por ejemplo, Henry et al. (2008) observó que la adición de diferentes proporciones de azúcares, ácidos orgánicos y aminoácidos a microcosmos de suelo resultaba en ratios de producción de N_2O (producto de una desnitrificación incompleta) frente a N_2 (producto

de una desnitrificación completa) diferentes. Por otro lado, mientras que un incremento de la concentración de nitratos en el sedimento contribuye a una mayor abundancia y diversidad (estructural y funcional) de la comunidad microbiana (Xu et al., 2014), proporciones altas de amonio frente a nitratos pueden potenciar la nitrificación frente a la desnitrificación, favoreciendo la acumulación de nitritos, que son tóxicos para los microorganismos (Zhang et al., 2017).

ii) **pH.** Se considera el factor abiótico más importante que influye sobre la actividad, composición y biodiversidad de la comunidad microbiana. Por un lado, influye de manera directa aumentando la competitividad y/o reduciendo la proliferación de aquellos taxones que no pueden subsistir en ambientes con pHs por debajo de su rango de supervivencia (Lauber et al., 2009). Por otro lado, su influencia sobre la comunidad microbiana puede ser indirecta, ya que otros factores abióticos de los sedimentos como la disponibilidad de nutrientes, las características del carbono orgánico, la capacidad de intercambio catiónico, la especiación química de metales o la salinidad dependen del pH (Liu et al., 2015).

iii) **Potencial redox.** Las variaciones del potencial redox (Eh) en el sedimento están vinculadas a la presencia de compuestos que actúan como aceptores de electrones (Hunting y Kampfraath, 2013) y, por tanto, influye sobre la función metabólica y composición de las comunidades microbianas. Mientras que en las zonas óxicas (altos Eh) del sedimento existen condiciones de oxigenación favorables para la proliferación de microorganismos aerobios (p.ej. oxidantes de metano, amonio o sulfitos), en las zonas anaerobias (bajos Eh) predominan los microorganismos anaerobios estrictos (p.ej. metanogénicos), a los que el oxígeno molecular les resulta tóxico. Excepcionalmente, las condiciones de la interfase anóxica del sedimento, en la que están presentes otros aceptores de electrones en sustitución al oxígeno, sostienen el metabolismo de microorganismos facultativos (p.ej. desnitrificantes o reductores de sulfatos y sulfitos), conduciendo a un incremento de la diversidad metabólica microbiana (Hong et al., 2019).

iv) **Salinidad.** Es un factor decisivo ya que la composición y diversidad de la comunidad microbiana está restringida a aquellos taxones tolerantes o capaces de

resistir el estrés y proliferar en ambientes salinos. En efecto, los principales iones que aportan salinidad, entre otros, Na^+, Cl^- y HCO_3^-, son tóxicos para muchos microorganismos e inhiben el crecimiento microbiano (Yu et al., 2012). Por otro lado, la respuesta del metabolismo microbiano a cambios en la salinidad varía entre distintos grupos funcionales; por ejemplo, mientras que la actividad de la DNRA aumenta con la salinidad, la desnitrificación disminuye (Laverman et al., 2007).

v) **Tamaño de partícula y composición mineralógica.** La composición, biodiversidad y actividad de la comunidad microbiana está significativamente relacionada con el tamaño de partícula del sedimento (Sessitsch et al., 2001; Grandy et al., 2008). Por un lado, las arenas, caracterizadas por tamaños de partícula superiores a 150 µm, presentan una menor área superficial que los limos y arcillas, por lo que dificultan la adhesión de los microorganismos al sedimento (Legg et al., 2012; Wang et al., 2013). Por otro lado, la fracción fina del sedimento (<63 µm) tiene un mayor contenido de materia orgánica y minerales secundarios (óxidos e hidróxidos de Fe y Mn, minerales arcillosos o carbonatos) que les confiere una gran capacidad de intercambio iónico (Hardy and Cornu, 2006) y adsorción de nutrientes y/o contaminantes que regulan la actividad enzimática de las comunidades microbianas.

vi) **Humedad.** Es un factor que regula diversas características del sedimento como la disponibilidad y flujo de oxígeno, materia orgánica, nutrientes, así como el pH, la salinidad o la densidad aparente, por lo que tiene un profundo efecto tanto en la estructura como en la actividad de la comunidad microbiana (Li et al., 2017).

vii) **Metales.** El efecto de los metales sobre la comunidad microbiana varía en función de su toxicidad o esencialidad. Sin embargo, aunque algunos metales son micronutrientes necesarios para el metabolismo microbiano, un incremento excesivo de sus concentraciones en el sedimento puede derivar también en efectos adversos. Entre estos, se han observado disminuciones de la abundancia, biodiversidad y actividad, así como la introducción de genes de resistencia a metales que les confieren la capacidad de adaptarse a ambientes con factores estresantes como elevadas cargas de contaminación metálica (Chen et al., 2018). A su vez, la acumulación de los metales en el sedimento depende de numerosos procesos fisicoquímicos y biológicos que se

producen entre los diferentes compartimentos de un sistema acuático (Fig. 1.7). Estos procesos se ven afectados por muchos factores fisicoquímicos, entre los que destacan:

i) **Tamaño de partícula.** La elevada área superficial, así como su mayor contenido de materia orgánica y minerales secundarios confiere a las partículas más pequeñas una gran capacidad de intercambio iónico y adsorción de metales (Hardy and Cornu, 2006).

ii) **pH.** Su influencia sobre los procesos de adsorción-desorción y solubilización-precipitación de metales en el sedimento estriba en la dependencia de la solubilidad y la carga superficial de los carbonatos, óxidos e hidróxidos de Fe y Mn, materia orgánica o arcillas con el pH. De manera general, la liberación de metales (o transformación de compuestos de menos a más solubles) aumenta con la reducción del pH (Eggleton y Thomas, 2004).

iii) **Potencial Redox.** Este parámetro influye significativamente en los procesos de oxidación-reducción y, en consecuencia, en la distribución química de los metales en el sedimento. Por ejemplo, la oxidación de la materia orgánica o sulfatos puede derivar en la liberación de los metales asociados y posterior precipitación como compuestos más solubles; entre otros, carbonatos, óxidos-hidróxidos de Fe y Mn o sulfuros metálicos (Kelderman y Osman, 2007).

iv) **Carbonatos, óxidos-hidróxidos de Fe y Mn, materia orgánica y composición mineralógica.** Los carbonatos, óxidos-hidróxidos de Fe y Mn, la materia orgánica y ciertos minerales como la arcilla (aluminosilicatos hidratados), confieren al sedimento una gran capacidad de intercambio iónico y, por tanto, contribuyen a la movilización de metales. Requieren una mención especial las sustancias húmicas y fúlvicas, que poseen una alta capacidad de interacción con iones libres, óxidos-hidróxidos, otros compuestos orgánicos y minerales para formar diversos complejos metálicos de estabilidad variable (Schnitzer, 1995). Por el contrario, minerales como el cuarzo confieren al sedimento una escasa capacidad de adsorción de metales (Horowitz, 1991).

1.3.3. Sustancias prioritarias y contaminantes específicos: contenido total, especiación química y bioaccesibilidad de metales

Los metales se consideran sustancias contaminantes cuya concentración debe vigilarse debido a su persistencia en el medioambiente, su capacidad para incorporarse en la cadena trófica y su toxicidad (Prosi, 1981; Armitage et al., 2007). La evaluación del riesgo toxicológico asociado a la presencia de metales en el medio ambiente debe fundamentarse en los siguientes cinco principios básicos (Fairbrother et al., 2007):

1) Los metales son constituyentes naturales del medioambiente cuyas concentraciones de fondo deben considerarse. Estas varían a lo largo de las diferentes regiones geográficas no sólo por la distinta composición de los suelos sino también por la acción de diversos procesos naturales y las interacciones entre los compartimentos ambientales. A su vez, estas concentraciones pueden verse alteradas por fuentes antropogénicas que introducen metales en los sistemas acuáticos (Fig. 1.7).

2) Los metales se encuentran de manera natural en los ecosistemas como mezclas de distintos elementos que presentan efectos aditivos, sinérgicos y/o antagónicos que deben examinarse.

3) Algunos metales son esenciales para el mantenimiento de una buena salud de los microorganismos, plantas, animales y seres humanos; por tanto, no sólo su exceso puede derivar en efectos no deseados sino también su deficiencia.

4) A diferencia de las especies orgánicas, los procesos químicos y biológicos (Fig. 1.8) no crean ni destruyen metales, sólo los transforman en otras especies (alterando su estado de oxidación) o formas orgánicas e inorgánicas.

5) La absorción, distribución, transformación y excreción de un metal en los seres vivos depende del tipo de metal, su forma química y la capacidad del organismo para regularlo o almacenarlo (Fig. 1.3). Es decir, el nivel ecotoxicológico de un metal viene determinado tanto por su disponibilidad química o medioambiental como por su biodisponibilidad (Michalke, 2003; Pagnanelli et al., 2004; Clozel et al., 2006; Liu y Zhao, 2007).

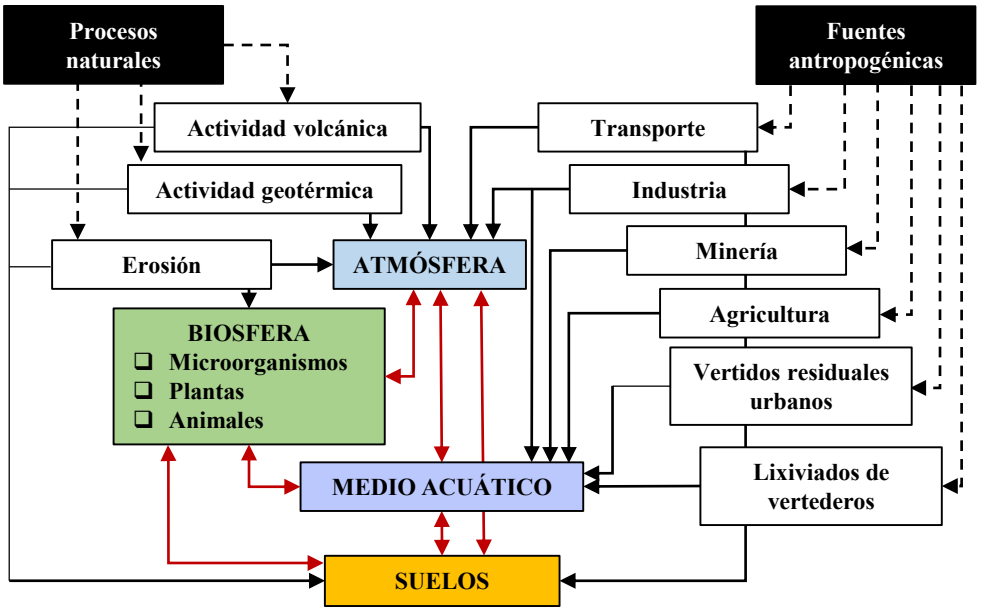

Fig. 1.7. *Diagrama descriptivo de las interacciones entre los compartimentos ambientales (flechas rojas), los procesos naturales y las fuentes antropogénicas (flechas negras) que influyen en el devenir y la concentración de los metales en los sistemas acuáticos. Fuente: Figura modificada de Gaillardet et al., 2014*

Se considera especiación química o medioambiental al conjunto de formas químicas en las que un contaminante se encuentra en una matriz y que determina su disponiblidad o capacidad de transferirse de un compartimento a otro dentro del sistema acuático. En el caso de los metales, estos pueden encontrarse libres o asociados a distintas fases orgánicas e inorgánicas (carbonatos, óxidos e hidróxidos de Fe y Mn o minerales primarios y secundarios) dependiendo de las caracterísitcas biológicas y fisicoquímicas del sedimento (Tessier et al., 1979; Namieśnik y Rabajczyk, 2010). Por otro lado, la biodisponibilidad se entiende como la fracción de un contaminante que puede interactuar con un organismo biológico e incorporarse a su estructura. Como parte de la biodisponibilidad, la bioaccesibilidad se define como la cantidad de contaminante que está disponible para su solubilización en el organismo sin que implique necesariamente su absorción por el sistema circulatorio. Por consiguiente, la toxicidad de un metal asociado al sedimento sobre la salud humana depende de: (1) su disponibilidad o especiación química en el sedimento; (2) su bioaccesibilidad; (3) su absorción por el sistema

circulatorio; y (4) su acumulación o excreción (Lanno et al., 2014; Harmsen, 2007). Nótese que las etapas (1), (2) y (3) determinan la biodisponibilidad del metal en el sedimento.

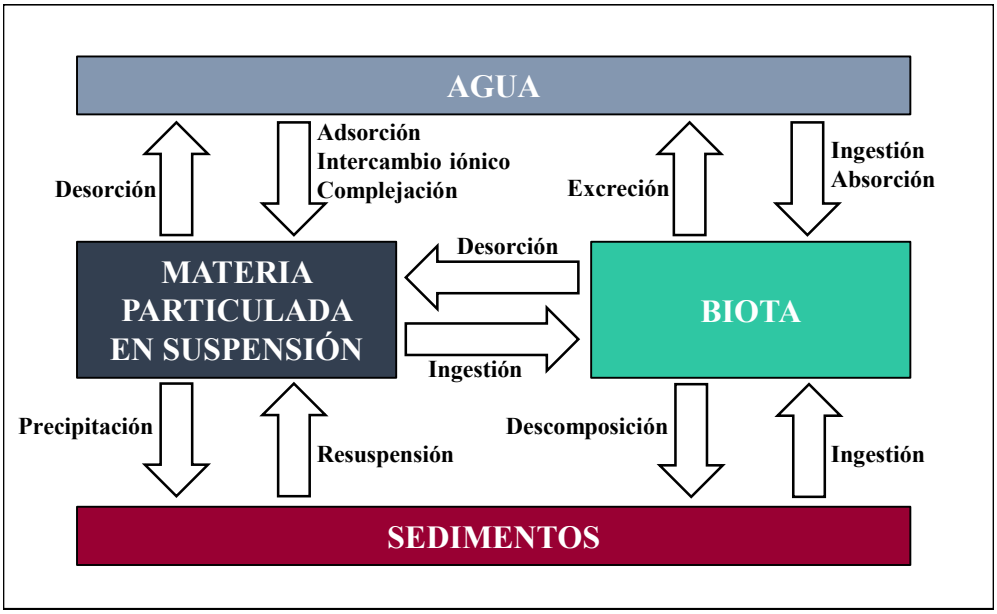

Fig. 1.8. *Principales procesos químicos y biológicos por los que los metales se transfieren entre los distintos compartimentos de un sistema acuático. Fuente: Figura modificada de Díaz de Alba, 2019*

En consecuencia, es razonable considerar no sólo el estudio de la **concentración total** sino también de la **especiación química** y la **bioaccesibilidad** como indicadores químicos de la toxicidad tanto ecológica como humana derivada de un exceso (o deficiencia) de metales en los sedimentos de una cuenca.

En este contexto, tanto la legislación europea (Directiva 2013/39/UE) como la estatal (Real Decreto 817/2015), por la que se establecen las normas de calidad ambiental (NCA) para su seguimiento, presentan la misma concepción errónea. Expresan estas NCA —concentración de un determinado contaminante en el agua, los sedimentos o la biota, que no debe superarse en aras de la protección de la salud humana y el medio ambiente— únicamente en función de su contenido total en la matriz en cuestión. Sin embargo, en el área académica se han empleado numerosos índices para determinar la potencialidad tóxica de los sedimentos con relación a su contenido en metales. Pueden dividirse en tres grandes grupos:

1) Índices basados en el **contenido total de metal** en el sedimento como indicativo del grado de desviación frente a concentraciones naturales de referencia, como la composición de la corteza terrestre superior o valores límite cuyos efectos adversos sobre los organismos bentónicos son conocidos. Destacan el Índice de Geoacumulación (en inglés, Geoaccumulation Index (Igeo)) (Nowrouzi y Pourkhabbaz, 2014), el Factor de Enriquecimiento (en inglés, Enrichment Factor (EF)) (Kaushik et al., 2019), el Índice de Carga Contaminante (en inglés, Pollution Load Index (PLI)) (Banu et al., 2013), el Índice de Riesgo Potencial Ecológico (en inglés, Potential Ecological Risk Index (PERI)) (Kabir et al., 2011), el Intervalo de Efecto Bajo y Mediano (en inglés, Effect Range Low and Median (ERL/ERM)) (Tokati, 2017) y el Nivel de Efecto Umbral y Probable (en inglés, Threshold and Predicted Effects Level (TEL/PEL)) (Zheng et al., 2008).

2) Índices basados en la **especiación química** del metal en el sedimento como indicativo del grado de movilidad o disponibilidad medioambiental. Destacan el Factor de Contaminación Individual y Global (en inglés, Individual and Global Contamination Factor (ICF/GCF)) (Saleem et al., 2015).

3) Índices basados en la **bioaccesibilidad** del metal en el sedimento como estimador de su biodisponibilidad. Destaca el Cociente de Peligrosidad (en inglés, Hazard Quotient (HQ)) desarrollado por la Agencia de Protección Ambiental de Estados Unidos (en inglés, United States Environmental Protection Agency (USEPA, 1993)) para la evaluación del riesgo sobre la salud humana debido a la exposición (ingesta, inhalación o contacto dérmico) a sedimentos contaminados.

La norma internacional ISO 17402:2008 proporcionó una guía para la selección y aplicación de diversas metodologías para estimar la biodisponibilidad oral de metales (incluidos los metaloides) y contaminantes orgánicos (incluidos los compuestos organometálicos) presentes en suelos y sedimentos. Estos procedimientos se clasifican en:

1) **Métodos biológicos o *in vivo***, basados en la evaluación de la biodisponibilidad y el efecto toxicológico de un contaminante a partir de su exposición directa a organismos vivos.

2) **Métodos químicos o *in vitro***, alternativas éticas de los primeros para el estudio de los efectos potenciales y/o acumulación de contaminantes en organismos que deben protegerse, mediante la determinación de la bioaccesibilidad. Estos a su vez, pueden clasificarse en tres categorías (Tabla 1.4):

2.1) **Pruebas químicas simples.** Constan de una sola fase (fase gástrica) y utilizan exclusivamente reactivos químicos inorgánicos sin ningún acondicionamiento fisiológico.

2.2) **Pruebas fisiológicas simples.** También constan de una sola fase; por lo general, la fase gástrica. Utilizan pocos reactivos químicos, pero con acondicionamiento fisiológico.

2.3) **Pruebas fisiológicas complejas.** Constan de varias fases (salival, gástrica y/o intestinal) y requieren de un mayor número de reactivos, en particular, aquellos más complejos que se corresponden con los análogos gastrointestinales (ácidos orgánicos, enzimas, y sales biliares, entre otros).

Tabla 1.4. *Metodos químicos o in vitro proporcionados por la norma ISO 17402:2008) para la estimación de la bioaccesibilidad oral en sedimentos. Fuente: Propia*

Pruebas químicas simples	Pruebas fisiológicas simples	Pruebas fisiológicas complejas
❑TCLP (Toxicity Characteristic Procedure)	❑RBALP (Relative Bioaccessibility Leaching Procedure) ❑SBET (Simplified Bioaccessibility Extraction Test)	❑PBET (Physiologically Based Extraction Test) ❑IVG (In vitro gastro-intestinal method) ❑RIVM test ❑SHIME (Simulator of the Human Intestinal Microbial Ecosystem) ❑UBM (Unified BARGE Bioaccessibility Method) ❑DIN test ❑TMO (TNO gastrointestinal model) ❑US Pharmacopeia model ❑MB&SR (Mass Balance & Soil Recapture)

La directriz fundamental de todas las pruebas fisiológicas consiste en el contacto de la muestra de sedimento con la solución cuya composición representa las condiciones del aparato digestivo humano. Sin embargo, existe una serie de parámetros específicos que varían

en función de la elección del protocolo o procedimiento que se desea aplicar, y que puede influir en la estimación de la bioaccesibilidad (RECORD, 2012):

i) **Compartimentos fisiológicos y tiempo de residencia.** El grado de absorción de contaminantes es notablemente superior en el estómago y el intestino delgado y, por consiguiente, la mayoría de los métodos tratan de imitar, al menos, uno de estos dos compartimentos del tracto digestivo humano. Por otro lado, el tiempo de residencia, aunque no es un parámetro muy sensible, es específico de cada compartimento. Mientras que para el estómago es de una hora, la permanencia en el intestino delgado se extiende hasta las tres horas y media, aproximadamente.

ii) **pH.** Es el parámetro más influyente y varía de un método a otro y entre compartimentos. En el caso de los metales, su disolución se ve favorecida por pHs bajos, mientras que un pH cercano a la neutralidad fomenta su precipitación (Grøn y Andersen, 2003). Si bien el pH estomacal bajo condiciones de ayuno varía entre 1 y 4, el pH del intestino delgado aumenta desde 4 hasta 7,5.

iii) **Temperatura.** Tanto la actividad enzimática como las propiedades fisicoquímicas del sedimento y los contaminantes dependen de este parámetro. En general, los protocolos establecen una temperatura de 37 °C.

iv) **Composición de las soluciones digestivas.** Generalmente, una mayor complejidad de las soluciones digestivas deriva en una simulación más representativa de las condiciones fisiológicas humanas. Al igual que los jugos gástricos, la mayoría de las soluciones estomacales empleadas están constituidas por HCl. Sin embargo, algunos protocolos incluyen la pepsina, enzima encargada de hidrolizar las proteínas y que, en el caso de los metales, aumenta su solubilidad mediante complejación (Grøn, 2005). Por otro lado, todas las soluciones intestinales están constituidas por $NaHCO_3$ (que aumenta el pH tras la fase estomacal) y por pancreatina (excepto el método MB&SR). Otros protocolos incluyen otras enzimas que incrementan la capacidad hidrolítica (lipasa o tripsina) o las propiedades tensioactivas (porcina o bovina como sustitutivos de la bilis humana) de las soluciones intestinales (Ng et al., 2010).

v) **Relación Sólido:Líquido.** Se considera otro factor influyente en la determinación de la bioaccesibilidad. En el caso del estudio de toxicidad en niños, estas relaciones varían entre 1:90 y 1:1125 (Oomen et al., 2006). Sin embargo, aunque una mayor relación Sólido:Líquido favorece la disolución de metales (Koch et al., 2013), trabajar con ratios próximos a 1:1000 dificulta la determinación de la bioaccesibilidad debido a los límites de cuantificación de los métodos analíticos.

vi) **Condiciones aerobias o anaerobias.** Estas condiciones regulan las reacciones de reducción-oxidación en el tracto digestivo. Mientras que el estómago funciona bajo condiciones aerobias, en el intestino prevalecen las condiciones anaerobias (Grøn, 2005).

vii) **Adición de comida.** El estado de ayuno se considera la condición más adecuada para el estudio de la bioaccesibilidad de elementos inorgánicos ya que induce pHs inferiores a los asociados al estado de digestión, incrementando así la disolución de contaminantes (Cave, et al., 2010). Por otro lado, el contenido en grasas y proteínas de los alimentos afecta significativamente a la bioaccesibilidad (Ng et al., 2010).

viii) **Método de agitación.** A fin de simular el peristaltismo o movimiento de contracción del tubo digestivo para la transición de los alimentos, todos los protocolos incorporan distintos tipos de agitación. La rotación vertical se considera más eficiente que la agitación horizontal (Oomen et al., 2002).

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2

Zona de estudio

Fuente: Propia

2. Zona de estudio

2.1. Descripción

La cuenca del río Deba, con una superficie total de 538 km^2, se extiende íntegramente por la Comunidad Autónoma del País Vasco (noroeste de España), perteneciendo en su mayoría al Territorio Histórico de Gipuzkoa (Fig. 2.1). Con un cauce de alrededor de 60 km, el río Deba nace en las regatas de Leintz-Gatzaga, próximo a las salinas. Aguas abajo del municipio de Arrasate recibe las aportaciones del afluente Oñati, a partir de donde discurre en dirección norte hasta desembocar en el mar Cantábrico (Golfo de Bizkaia), después de la confluencia con el afluente Ego, principal tributario en la parte baja de la cuenca. Localizada en la Cornisa Cantábrica, la cuenca se eleva desde el nivel del mar (0 m, Golfo de Bizkaia) hasta los 1320 m (pico Botreaitz), presentando una pendiente máxima del 40% en la zona alta (Fig. 2.1).

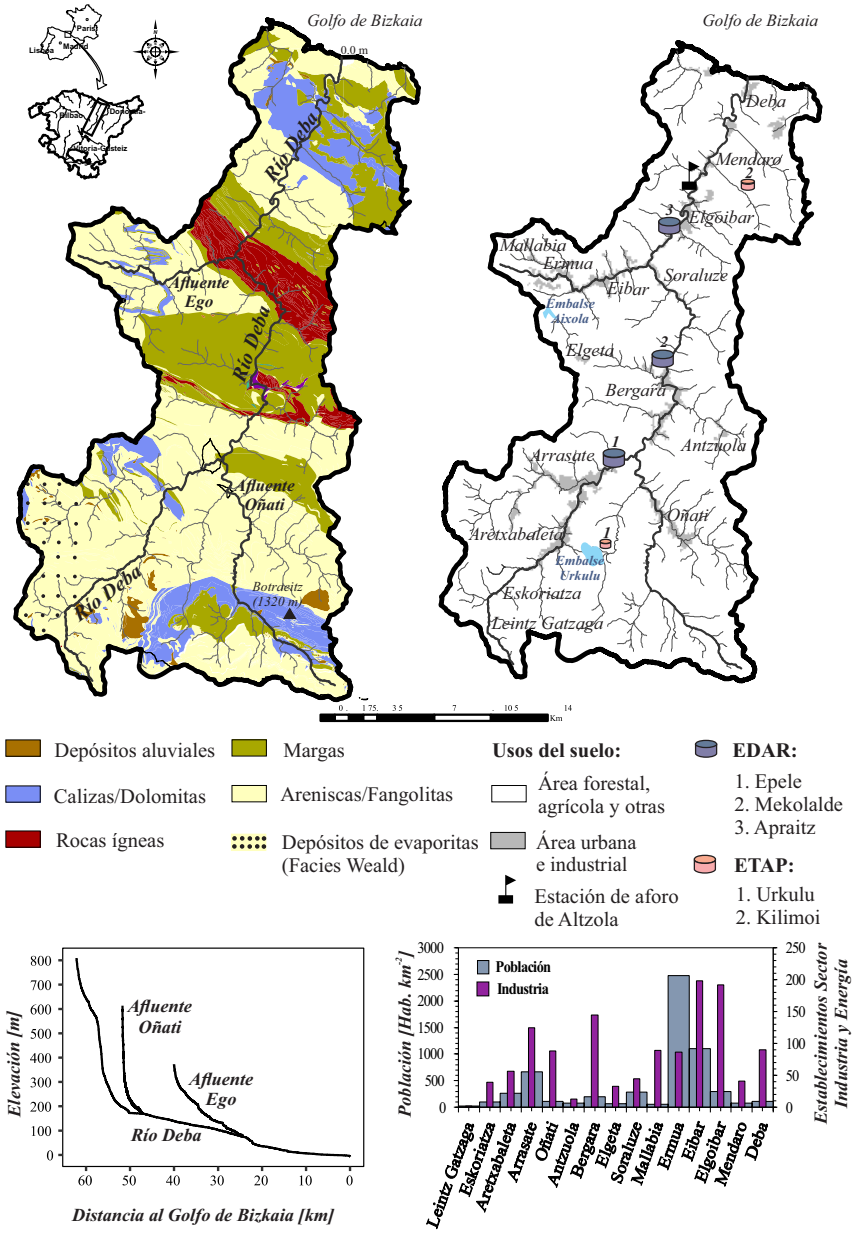

Fig. 2.1. *Localización geográfica, litología, usos del suelo y perfil de alturas de la cuenca del río Deba. Fuente: Elaboración propia a partir de datos obtenidos del Geoportal de referencia de la Infraestructura de Datos Espaciales de Euskadi (www.geo.esukadi.eus). Se incluyen los embalses, principales municipios, estaciones depuradoras de aguas residuales (EDARes), estaciones de tratamiento de agua potable (ETAP) y la estación de aforo de Altzola. Se adjunta como gráfica de barras la distribución espacial de la población (Habitantes por km²) y del número de establecimientos ligados al sector de la Industria y Energía, en los principales municipios. Fuente: Elaboración propia a partir de los últimos datos reportados por el Instituto Vasco de Estadística – Euskal Estatistika Erakundea (www.eustat.eus)*

2.1.1. Geología y litología

El lecho rocoso de la cuenca consiste en su mayoría en una sucesión de rocas sedimentarias de edad mesozoica, concretamente del Cretácico Superior e Inferior (IGME, 2015). Mientras que en la zona sur predominan las areniscas y fangolitas, en la zona central y norte destacan las margas y las rocas carbonatadas (calizas y dolomitas) (Fig. 2.1).

Cabe destacar dos rasgos geológicos significativos en la cuenca del río Deba:

3) La concurrencia en la zona alta de depósitos de evaporitas (yesos y anhidritas) en alternancia con areniscas, fangolitas, arcillas y alguna caliza, característico de las Facies Weald del Cretácico Inferior (EVE, 1989). Concretamente, en el municipio de Leintz-Gatzaga (Fig. 2.1), reconocido por la existencia de unas importantes salinas, las evaporitas se encuentran como estructuras diapíricas (Martínez-Santos et al., 2015). Aunque su presencia no es visible en superficie, el agua sulfurosa que emerge de los manantiales de Eskoriatza y Aretxabaleta, con altos contenidos de Na^{+}, Cl^{-}, SO_4^{2-} y H_2S disuelto, da una idea de las características del terreno por el que discurre (Iribar y Ábalos, 2011).

4) La presencia de rocas ígneas en la zona de confluencia del afluente Ego con el río Deba (Fig. 2.1), como producto volcánico del Cretácico Superior del Sinclinorio de Vizcaya (ITME, 1989).

2.1.2. Cobertura y usos del suelo

Los Cambisoles (66%) y Acrisoles (25%) son los tipos de suelos que predominan en la cuenca del río Deba. El 93% de la superficie total no urbana o industrial pertenece a uso forestal, donde un 75% lo constituyen superficies arboladas o bosques, en las que destacan las plantaciones de la especie *Pinus* spp (59%). De la superficie no forestal, sólo un 6% es de uso agrícola (EJ-GV, 2018).

La actividad económica de la cuenca está muy ligada al sector industrial, concentrada en los grandes núcleos urbanos ubicados a lo largo del río, especialmente en Eibar, Elgoibar,

Arrasate y Bergara (Fig. 2.1). Las principales fábricas pertenecen a la industria metalúrgica, automotiva, galvanizadora, siderúrgica y de la construcción (maquinaria y electrodomésticos).

2.1.3. Climatología e hidrología

Como parte de la red hidrometeorológica de la Comunidad Autónoma Vasca, la Diputación Foral de Gipuzkoa – Gipuzkoako Foru Aldundia dispone de tres estaciones permanentes de aforo y calidad de las aguas en la cuenca del río Deba. Además de un pluviómetro y una regleta ubicada en el cauce natural, estas estaciones poseen un limnígrafo, un limnímetro digital y una sonda piezorresistiva de nivel situados en la caseta de mediciones, donde un pozo comunica el lecho del río con la zona de medición. De esta manera, registran y transmiten cada 10 minutos datos meteorológicos e hidrológicos que pueden consultarse en tiempo real (www.gipuzkoa.eus). Conjuntamente, estas estaciones constan de una serie de sensores y equipos que miden algunos de los parámetros fundamentales para la determinación de la calidad del agua del río, como temperatura, oxígeno disuelto, conductividad eléctrica, pH, turbidez, nutrientes y materia orgánica. La estación de aforo y calidad del agua de Altzola (Fig. 2.1), situada cerca de la salida de la cuenca, concretamente en el municipio de Elgoibar, recopila desde 1996 datos meteorológicos e hidrológicos correspondientes a una superficie drenante de 464,25 km^2 (alrededor del 86% de la superficie total de la cuenca).

La precipitación y temperatura anual media registrada durante la última década (2008 – 2018) fueron de 1425 mm y 13,7°C, respectivamente. El periodo más frío y lluvioso se extendió desde noviembre hasta marzo, mientras que la época más seca y calurosa tuvo lugar durante el verano, de junio a septiembre (Fig. 2.2). En cuanto al caudal, la media anual durante la última década (2008 – 2018) fue de 12.3 $m^3\ s^{-1}$, registrándose caudales mensuales medios superiores a 15 $m^3\ s^{-1}$, desde noviembre hasta marzo.

El periodo en el que se centró este trabajo (de enero 2015 a enero 2016), se caracterizó por ser una época de precipitación y temperatura medias levemente inferiores a las registradas en la última década (1385 mm y 13,2°C, respectivamente). Sin embargo, un periodo aún más seco lo constituyeron los años hidrológicos 2009/2010, 2010/2011 y 2011/2012, en los que se registró una precipitación anual media de 1200, 1155 y 1205 mm, respectivamente (Fig. 2.2). Asimismo, los eventos de mayor caudal acontecieron en los meses de febrero

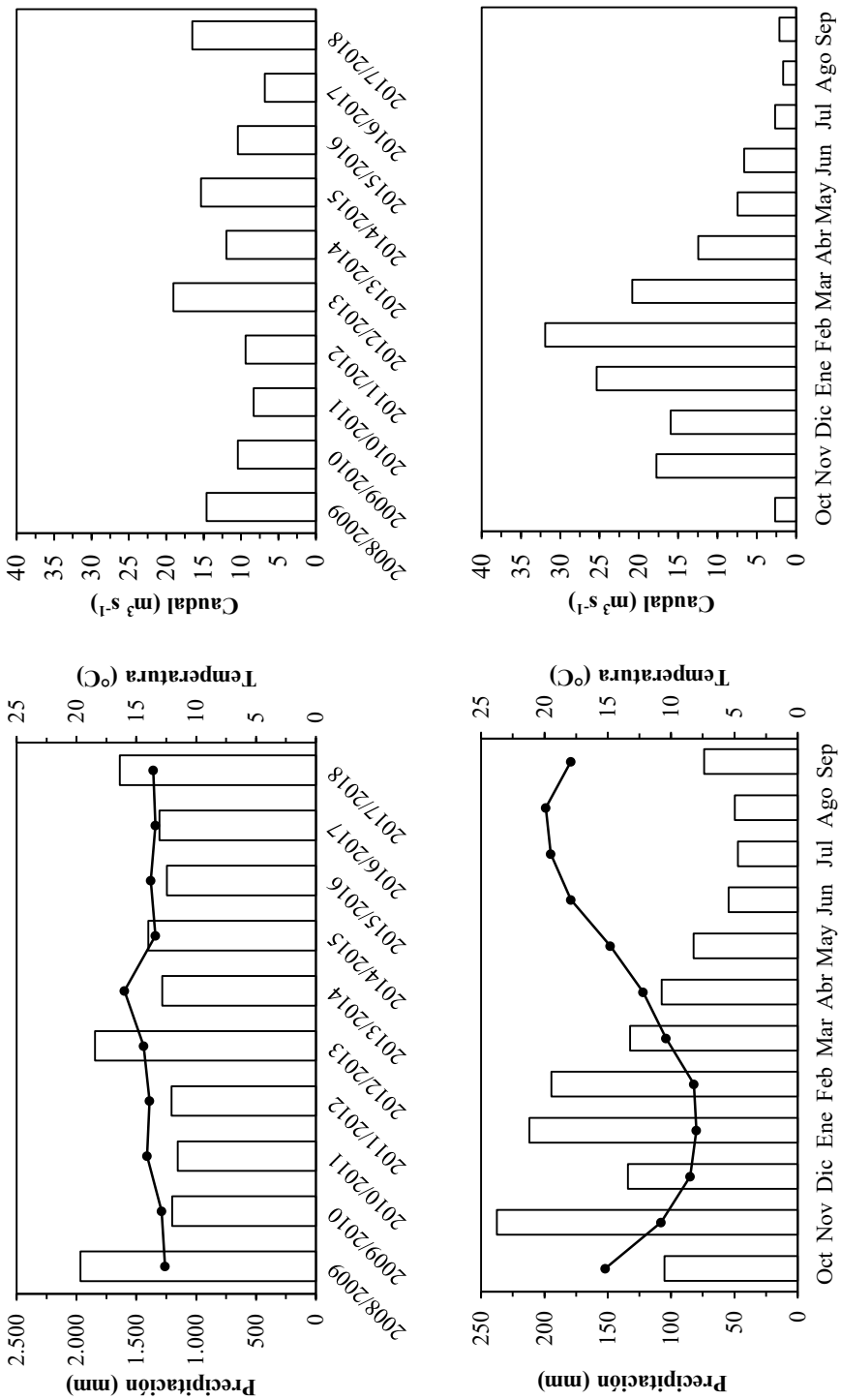

Fig. 2.2. *Medias anuales y mensuales de la precipitación (gráfico de barras), temperatura (gráfico lineal) y caudal, medidas en la estación de aforo de Altzola durante la década que comprende los años hidrológicos desde 2008/2009 hasta 2017/2018. Fuente: Elaboración propia a partir de datos reportados por la Diputación Foral de Gipuzkoa – Gipuzkoako Foru Aldundia (www.gipuzkoa.eus)*

y, especialmente, enero de 2015, registrándose un caudal diario de 423 m^3 s^{-1} y un caudal máximo instantáneo de 438 m^3 s^{-1} durante la crecida del 30 de enero de 2015 (Fig. 2.3). Al contrario que en la última década (2008 – 2018), la época de aguas bajas se extendió hasta finales de noviembre de 2015.

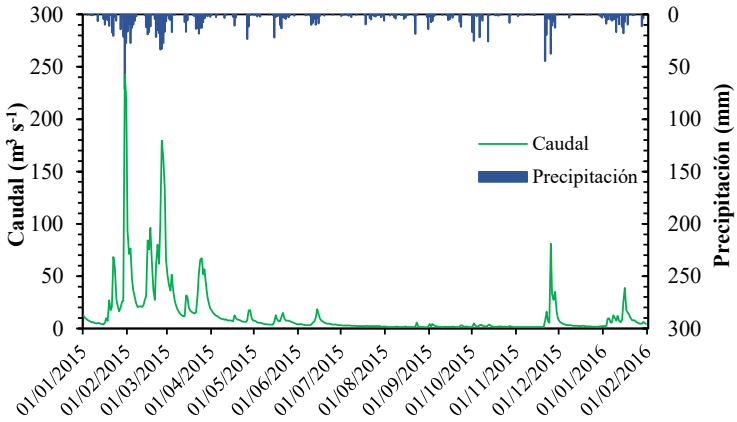

Fig. 2.3. *Hidrograma para el periodo de estudio con valores de precipitación y caudal diarios registrados en la estación de aforo de Altzola. Fuente: Elaboración propia a partir de datos reportados por la Diputación Foral de Gipuzkoa – Gipuzkoako Foru Aldundia (www.gipuzkoa.eus)*

2.1.4. Abastecimiento y saneamiento de aguas

En la cuenca existen numerosos sistemas de abastecimiento de aguas. Entre las diversas fuentes de captación (e.g. manantiales, vaguadas, sondeos o captaciones superficiales) destacan:

a) El embalse de Urkulu (Fig. 2.1), con una capacidad de 10 hm^3, abastece a los municipios de Eskoriatza, Aretxabaleta, Arrasate, Oñati, Bergara, Elgeta, Soraluze y Antzuola.

b) El embalse de Aixola (Fig. 2.1), con una capacidad de 2,73 hm^3, abastece a los municipios de Ermua y Eibar.

Mientras que en algunos sistemas, las aguas captadas son directamente conducidas a los municipios para el abastecimiento de su población, otros, incluyen una estación de tratamiento de agua potable (ETAP). Entre las más importantes:

a) La ETAP de Urkulu (Fig. 2.1), ubicada en el municipio de Aretxabaleta, fue puesta en funcionamiento en 1995 y tiene una capacidad de abastecimiento de 105,000 habitantes (Eskoriatza, Aretxabaleta, Arrasate, Oñati, Bergara, Elgeta, Soraluze y Antzuola).

b) La ETAP de Kilimoi (Fig. 2.1), ubicada en el municipio de Mendaro, fue puesta en funcionamiento en 1991 y tiene una capacidad de abastecimiento de 30,000 habitantes (Elgoibar, Mendaro y Deba).

A finales de los años 90, se diseñó un proyecto de implantación de medidas correctoras relacionadas con el saneamiento y depuración de las masas de agua, que consistía principalmente en la construcción de cuatro estaciones depuradoras de aguas residuales (EDARes), gestionadas por el Consorcio de Aguas de Gipuzkoa – Gipuzkoako Urak. Antes de la puesta en marcha de estas instalaciones, las aguas residuales urbanas e industriales eran vertidas directamente a los cauces del río y al mar:

a) La EDAR de Arronamendi, ubicada en Deba, fue puesta en marcha en 1998 y da servicio a este municipio. El agua depurada se vierte directamente al mar (Golfo de Bizkaia), por lo que no ha sido incluida en la Fig. 2.1.

b) La EDAR de Apraitz, ubicada en el municipio de Elgoibar (Fig. 2.1), fue puesta en funcionamiento en 2007 y da servicio a los municipios de Soraluze, Mendaro y Elgoibar. El vertido directo de las aguas residuales procedentes de los municipios de Ermua y Eibar al afluente Ego, cesó con la construcción del colector Ermua – Eibar y su posterior conexión a la depuradora en junio de 2014. Por otro lado, parte de las aguas residuales de Mallabia se depuran en una pequeña EDAR localizada en el propio municipio. En la actualidad, la Agencia Vasca del Agua – Ur Agentzia dirige la construcción de un nuevo tramo de colector con el que se pretende conectar el saneamiento íntegro de esta zona a la red de Ermua y, finalmente, a la EDAR de Apraitz (URA, 2018b).

c) La EDAR de Mekolalde (Fig. 2.1), ubicada en el municipio de Bergara, fue puesta en funcionamiento en 2009 y da servicio a los municipios de Antzuola, Elgeta y Bergara.

d) La EDAR de Epele (Fig. 2.1), ubicada en el municipio de Arrasate, fue puesta en marcha en 2012 y da servicio a los municipios de Eskoriatza, Aretxabaleta, Arrasate y Oñati.

En la Tabla 2.1, se describen los procesos de depuración que conforman la línea de aguas en las EDARes antes mencionadas. Debe destacarse que durante el periodo comprendido entre el 1 de mayo y el 15 de octubre, se incluye en las estaciones de Apraitz, Mekolalde y Epele una etapa de eliminación química del fósforo con $FeCl_3$ (sin adición de CaO), de manera que la concentración máxima de PO_4^{3-} en los efluentes de salida no supere los 3 ppm. Además, las EDARes no están diseñadas para la eliminación de metales, por lo que sólo se recolectan aquellas aguas residuales cuyo contenido metálico no inhiba la actividad de los microorganismos en el tratamiento secundario.

Tabla 2.1. *Especificaciones y procesos que constituyen la línea de aguas de las EDARes en la cuenca del río Deba. Fuente: Elaboración propia a partir de la información reportada por el Consorcio de Aguas de Gipuzkoa – Gipuzkoako Urak (www.gipuzkoakour.eus)*

	Especificaciones		**Pretratamiento**			**Tratamiento primario**	**Tratamiento secundario**				
EDAR	**Habitantes equivalentes**	**Caudal entrada ($m^3\ h^{-1}$)**	**Desbaste**	**Desarenado**	**Desengrasado**	**Decantación primaria**	**Reactor flujo pistón**	**Aireación prolongada**	**Reactor biológico secuencial (SBR)**	**Desgasificador**	**Decantación secundaria**
Arronamendi	5,000	250–350									
Apraitz	95,000	5623									
Mekolalde	35,000*	2340									
Epele	90,000	3053									

(*) Opera a la mitad de su capacidad.

2.2. Presiones antrópicas

Antiguamente, el río Deba fue navegable hasta Altzola, donde existió un importante puerto comercial, clave en la exportación de lana e importación de mineral de hierro con Inglaterra durante los siglos XV y XVII. Posteriormente, la calidad de sus aguas sufrió las consecuencias de la intensa industrialización que se produjo a mediados del siglo XX en

los municipios de la cuenca. El aumento poblacional debido al desarrollo industrial que se produjo, y que no fue regulado, generó una gran contaminación de todo el río y sus afluentes.

En este contexto, el Plan Hidrológico de la Demarcación Hidrográfica del Cantábrico Oriental, correspondiente al primer ciclo de planificación (2009 – 2015), fue aprobado mediante el Real Decreto 400/2013, de 7 de junio, por requerimiento legal establecido por la Directiva Marco del Agua (2000/60/CE) y su transposición a la legislación española. En la actualidad, se encuentra derogado y en etapa de revisión por el segundo ciclo de planificación (2015 – 2021). Según esta planificación, la demarcación hidrográfica del Cantábrico Oriental está dividida en sistemas de explotación y unidades hidrológicas (UH), entre las que se encuentra la UH Deba (Fig. 2.4), cuya planificación gestiona la Agencia Vasca del Agua – Ur Agentzia en el ámbito de la demarcación competencia de la Comunidad Autónoma del País Vasco (URA, 2012).

Fig. 2.4. *División de la demarcación hidrográfica del Cantábrico Oriental. Fuente: Plan Hidrológico de la Demarcación Hidrográfica del Cantábrico Oriental (2009-2015)*

Para el cumplimiento de los objetivos de la planificación hidrológica, entre los que se encuentran la consecución del buen estado de las aguas y la adecuada protección del medio ambiente, así como su armonización con la satisfacción de las demandas de agua y el

desarrollo regional y sectorial, la Agencia Vasca del Agua – Ur Agentzia elaboró inicialmente un inventario de presiones sobre el medio hídrico (URA, 2012). Las presiones antropogénicas a las que están expuestas las masas de agua, se identificaron y cuantificaron atendiendo a la siguiente clasificación:

a) Presión ejercida por fuentes puntuales de contaminación.

b) Presión ejercida por fuentes de contaminación difusa.

c) Presiones hidromorfológicas.

d) Presiones biológicas.

Tabla 2.2. *Datos fisicoquímicos, aportados por el Consorcio de Aguas de Gipuzkoa – Gipuzkoako Urak, de los efluentes tratados en las EDARes que vierten sobre el cauce principal de la cuenca del río Deba para el periodo de estudio (enero 2015 – enero 2016). Número de muestreos (N), valores medios y rangos (mínimo – máximo) del pH, conductividad eléctrica (CE), sólidos suspendidos (SS), nutrientes (NH_4^+, NO_3^- y P total), demanda biológica y química de oxígeno (DBO_5 y DQO, respectivamente), y metales disueltos (Fe, Mn, Zn, Cu, Cr, Ni y Pb). Los límites de detección (< l.d.) fueron: 5 mg L^{-1} para los SS, 0,5 mg L^{-1} para N-NH_4^+ y P total, 2 mg L^{-1} para N-NO_3^-, 5 mg L^{-1} O_2 para DBO_5, 10 mg L^{-1} O_2 para DQO, 0,5 mg L^{-1} para Fe y 0,25 mg L^{-1} para el resto de metales disueltos*

	EDAR Epele			**EDAR Mekolalde**			**EDAR Apraitz**		
Parámetro	*N*	*Media*	*Rango*	*N*	*Media*	*Rango*	*N*	*Media*	*Rango*
pH	24	7,3	6,9-7,5	26	7,3	7,1-8,3	22	7,0	6,7-7,2
CE [µS cm^{-1}]	24	664	379-771	26	556	306-678	22	703	329-908
SS [mg L^{-1}]	22	15,0	<l.d.-45,0	26	7,3	<l.d.-30,0	22	12,3	<l.d.-42,0
NH_4^+ [mg N L^{-1}]	24	0,6	<l.d.-1,4	25	<l.d.	<l.d.-0,8	21	0,6	<l.d.-3,2
NO_3^- [mg N L^{-1}]	24	8,1	3,4-19,3	26	4,6	<l.d.-9,4	22	7,4	2,3-12,5
P total [mg L^{-1}]	24	1,5	<l.d.-3,8	26	1,8	<l.d.-0,5	22	2,0	<l.d.-5,5
DBO_5 [mg O_2 L^{-1}]	23	7,2	<l.d.-15	26	5,4	<l.d.-15,0	22	8,1	<l.d.-27,0
DQO [mg O_2 L^{-1}]	24	45	19-120	26	23	<l.d.-72	22	32	14-74
Fe [mg L^{-1}]	24	0,6	<l.d.-1,4	26	0,6	<l.d.-1,1	21	<l.d.	<l.d.
Mn [mg L^{-1}]	24	<l.d.	<l.d.	26	<l.d.	<l.d.	21	<l.d.	<l.d.
Zn [mg L^{-1}]	24	<l.d.	<l.d.	26	<l.d.	<l.d.	22	<l.d.	<l.d.
Cu [mg L^{-1}]	24	<l.d.	<l.d.	26	<l.d.	<l.d.	22	<l.d.	<l.d.
Cr [mg L^{-1}]	24	<l.d.	<l.d.	26	<l.d.	<l.d.	22	<l.d.	<l.d.
Ni [mg L^{-1}]	24	<l.d.	<l.d.	26	<l.d.	<l.d.	22	<l.d.	<l.d.
Pb [mg L^{-1}]	24	0,26	<l.d.	26	<l.d.	<l.d.	22	<l.d.	<l.d.

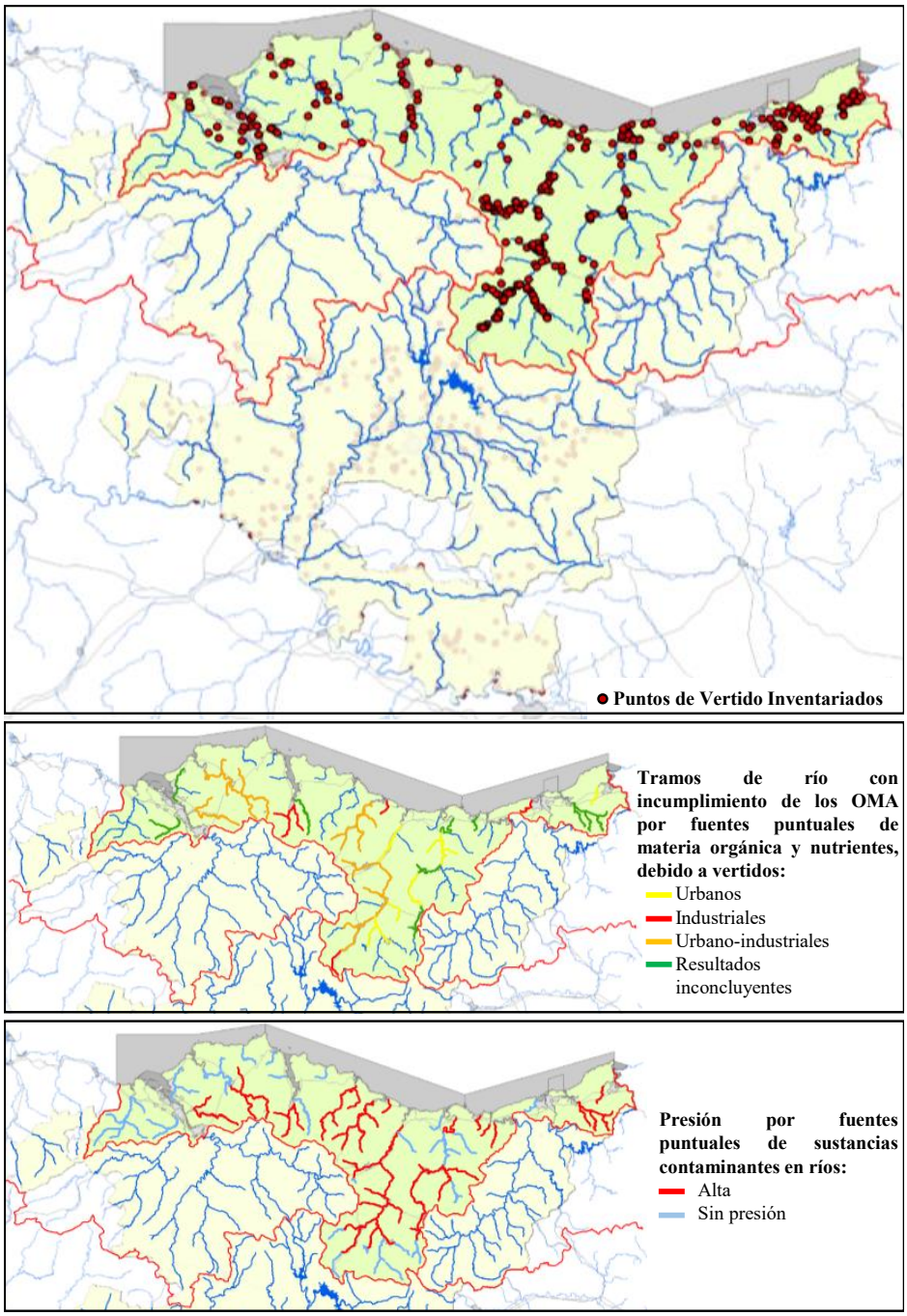

Fig. 2.5. *Localización de los vertidos urbanos e industriales inventariados para: (a) la identificación de las presiones por aportes de materia orgánica y nutrientes, y posterior evaluación del cumplimiento de los objetivos medioambientales (OMA); (b) la clasificación de la presión ejercida por sustancias contaminantes. Fuente: Figura modificada del informe de la Agencia Vasca del Agua – Ur Agentzia (URA, 2012)*

2.2.1. Fuentes de contaminación puntual

Las presiones ejercidas por fuentes de contaminación puntual de los ríos (Fig. 2.5) se deben a vertidos urbanos e industriales que se realizan directamente sobre los cauces, y que aportan materia orgánica, nutrientes y sustancias contaminantes (incluyen sustancias prioritarias, sustancias preferentes y otros contaminantes según el Real Decreto 817/2015) a las masas de agua.

Tabla 2.3. *Datos fisicoquímicos, aportados por la Diputación Foral de Gipuzkoa – Gipuzkoako Foru Aldundia, de los efluentes sin tratar que se vierten directamente sobre el cauce principal([1]) y los afluentes Oñati([2]) y Ego([3]), procedentes de las principales actividades industriales desarrolladas en los municipios de la cuenca del río Deba. Caudales volumétricos (Q) y másicos (sólidos suspendidos (SS), demanda química de oxígeno (DQO) y metales disueltos (Fe, Zn, Cr, Cu y Ni)) anuales. Fuente: Tabla modificada de Martínez-Santos et al., 2015*

Parámetro	**Eskoriatza[1]**	**Aretxabaleta[1]**	**Arrasate[1]**	**Oñati[2]**	**Bergara[1]**	**Soraluze[1]**	**Eibar[3]**	**Elgoibar[1]**
Q [m^3 $año^{-1}$]	90	648	1052	1262	336	1780	1530	75
SS [kg $año^{-1}$]	1167	59	2032	4905	3724	2133	2779	2217
DQO [kg O_2 $año^{-1}$]	514	114	1741	3365	2934	2947	2346	2075
Fe [kg $año^{-1}$]	178	1	63	487	539	6	78	13
Zn [kg $año^{-1}$]	1	1	14	61	544	58	8	
Cr [kg $año^{-1}$]	4		21	1	22		8	
Cu [kg $año^{-1}$]			1	3	1			
Ni [kg $año^{-1}$]	10		53	5	8	18	10	

Como se ha descrito previamente, el cauce principal recibe los efluentes tratados de tres EDARes localizadas a lo largo de la cuenca del río Deba (Fig. 2.1). El Consorcio de Aguas de Gipuzkoa – Gipuzkoako Urak, encargado de su gestión, caracteriza bimensualmente estos efluentes para determinar sus contenidos de materia orgánica, nutrientes y metales, entre otros parámetros fisicoquímicos (Tabla 2.2). Sin embargo, las aguas urbanas residuales de

municipios como Mallabia aún no son íntegramente tratadas y se vierten directamente al afluente Ego. Por otro lado, puesto que las EDARes no están diseñadas para la eliminación de metales, cerca de 6773 m^3 de vertidos industriales sin tratar son directamente vertidos sobre el cauce principal y sus afluentes (Tabla 2.3).

2.2.2. Fuentes de contaminación difusas

Las presiones ejercidas por fuentes de contaminación difusas se deben a la agricultura, la ganadería y los abonados orgánicos, así como los emplazamientos potencialmente contaminantes del suelo como vertederos, solares o emplazamientos industriales. Mientras que no se definieron presiones significativas con origen agrícola o emplazamientos potencialmente contaminantes, las presiones debidas a aportes de nitrógeno y fósforo procedentes de la ganadería y abonados orgánicos se clasificaron como moderados, e incluso altos, en algunas secciones del río Deba (Fig. 2.6).

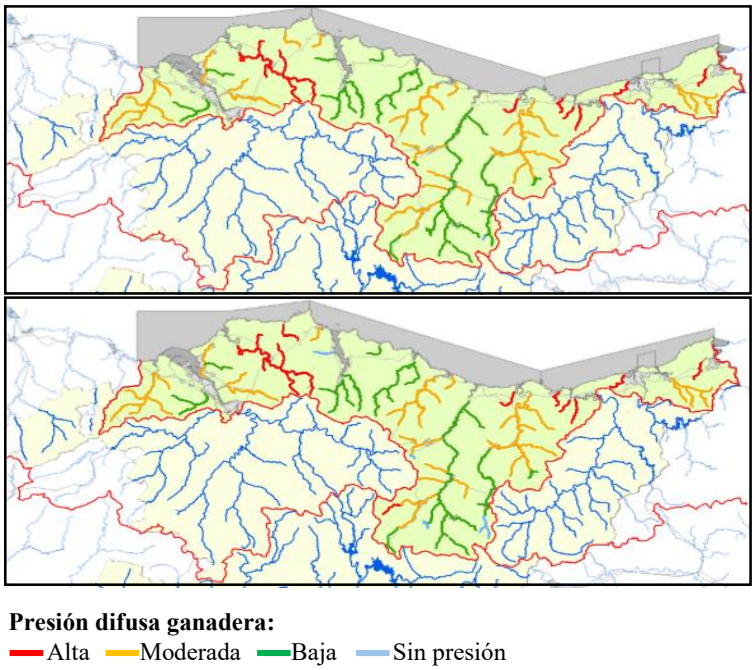

Fig. 2.6. *Presión por nitrógeno (superior) y fósforo (inferior) de origen ganadero sobre los ríos de la Comunidad Autónoma del País Vasco. Fuente: Figura modificada del informe de la Agencia Vasca del Agua – Ur Agentzia (URA, 2012)*

2.2.3. Presiones hidromorfológicas

Las presiones hidromorfológicas se deben a alteraciones físicas en el medio acuático, entre las que se incluyen:

i) Las presiones por regulación del régimen hidrológico de las presas. En la cuenca del río Deba, se observaron alteraciones significativas en los propios embalses (Urkulu y Aixola, Fig. 2.1) e incluso aguas abajo (afluentes Oñati y Ego hasta su confluencia con el río Deba, respectivamente). La capacidad reguladora de estos embalses oscila entre el 37% (Aixola) y el 69% (Urkulu) del volumen máximo embalsado.

ii) Las presiones por alteraciones morfológicas. A lo largo de la cuenca, el río sufre presiones morfológicas de nivel moderado e incluso fuerte, ejercidas por tramos cortados o desviados (1,3% de reducción), canalizaciones que afectan a más del 18% de los márgenes del río o coberturas, especialmente en el afluente Ego, ya que se encuentra cubierto en un 40% de su longitud total (URA, 2018a).

Tabla 2.4. *Demandas de agua (hm^3) anuales en el año 2012 y en los escenarios proyectados para los años 2015, 2021 y 2027. Los usos del agua considerados son: urbano (incluye uso doméstico, comercial, industrial y ganadero conectados a la red urbana, y riego), industrial y ganadero (abastecimiento mediante tomas propias), y otros usos (energético, acuicultura o usos recreativos, entre otros). Fuente: Agencia Vasca del Agua – Ur Agentzia (URA, 2012)*

	Urbano									
	Doméstica	Comercial	Industrial	Municipal	Riego privado	Ganadería	Total	**Industrial de toma propia**	**Ganadería rural**	**Otros usos**
2012	69,23	14,91	21,72	9,44	0,06	1,75	117,10	13,18	0,22	0,24
2015	78,52	15,74	28,24	9,88	0,08	1,83	134,28	13,18	0,22	0,24
2021	79,36	15,87	28,52	9,91	0,08	1,85	135,59	13,18	0,22	0,24
2027	79,09	15,80	28,53	9,86	0,08	1,85	135,21	13,18	0,22	0,24

iii) Las presiones por usos consuntivos del agua para abastecimiento de la población, siendo los embalses de Urkulu y Aixola, así como el afluente Ego hasta su confluencia con el río Deba, las masas de agua que presentaron alteraciones significativas. Tal y como se observa en la Tabla 2.4, después del uso doméstico, las actividades industriales

son las que mayor abastecimiento de agua exigieron en el año 2012 y se prevé que exijan (proyecciones para los años 2015, 2021 y 2027, tomando el año 2012 como referencia) en la cuenca del río Deba.

iv) Las presiones por detracción de caudal para el funcionamiento de las centrales hidroeléctricas (uso no consuntivo del agua), que ocasionan alteraciones significativas en la mayor parte del cauce principal (desde el municipio de Eskoriatza hasta Mendaro) y el afluente Oñati.

2.3. Estado ecológico

Desde principios de los años 90, la Agencia Vasca del Agua – Ur Agentzia tiene establecidos una serie de programas de seguimiento del estado ecológico de los ecosistemas fluviales, en el ámbito de la demarcación competencia de la Comunidad Autónoma del País Vasco, como elemento central para la consecución de los objetivos medioambientales planteados en los Planes Hidrológicos de la Demarcación Hidrográfica del Cantábrico Oriental. Dentro de estos programas de seguimiento, se evalúan la calidad biológica y fisicoquímica de las masas de agua superficial, que junto con el análisis de indicadores hidromorfológicos, determinan el estado o potencial ecológico de los ríos.

Tal y como se observa en la Fig. 2.7, de acuerdo con la red de seguimiento de la Agencia Vasca del Agua – Ur Agentzia , el eje principal del Deba está dividido en cuatro masas de agua: Deba-A (tramo alto), Deba-B (tramo medio), Deba-C y Deba-D (tramo bajo). Además, dentro de esta red están incluidas las masas de agua correspondientes a los principales afluentes, Oñati-A, Oñati-B (afluente Oñati) y Ego-A (afluente Ego), así como a otros afluentes menores y arroyos de la cuenca, como Aramaio-A (afluente Aramaio), Arantzazu-A (afluente Arantzazu, tributario del afluente Oñati), Angiozar-A (afluente Angiozar), Antzuola-A (afluente Antzuola), Ubera-A (afluente Ubera), Kilimoi-A (afluente Kilimoi) y Santuarran-A (arroyo Santuarran).

En la última campaña del 2018, todas las masas de agua presentaron un buen estado ecológico, excepto Deba-C, Ubera-A, Antzuola-A y Satuarran-A (Fig. 2.7). Del análisis interanual 2014 – 2018 del estado o potencial ecológico de las 14 masas de agua en la cuenca

del río Deba, se dedujeron 4 incumplimientos leves (Deba-A, Deba-C, Deba-D y Ubera-A) y 3 graves (Antzuola-A, Ego-A y Satuarran-A) de los objetivos medioambientales dentro del plazo previsto (URA, 2019a). Durante el quinquenio (Tabla 2.5), la masa Deba-A presentó un potencial ecológico insuficiente únicamente en 2015, debido a incumplimientos de indicadores biológicos. Por su parte, la masa Deba-B experimentó una considerable mejoría del estado biológico y fisicoquímico del agua, especialmente a partir del año 2016, cumpliendo así con el objetivo de alcanzar un buen estado ecológico al 2021. Por el contrario, la masa Deba-C presentó incumplimientos sistemáticos de los indicadores biológicos y, en ocasiones, de la calidad fisicoquímica de las aguas, por lo que aún no se cumple el objetivo de buen estado ecológico al 2021. Igualmente, aunque la masa Deba-D presentó un buen estado ecológico en la campaña de 2018, calidades insuficientes de los indicadores biológicos y fisicoquímicos del agua durante el resto del quinquenio ocasionaron el incumplimiento leve de los objetivos medioambientales en este tramo del río.

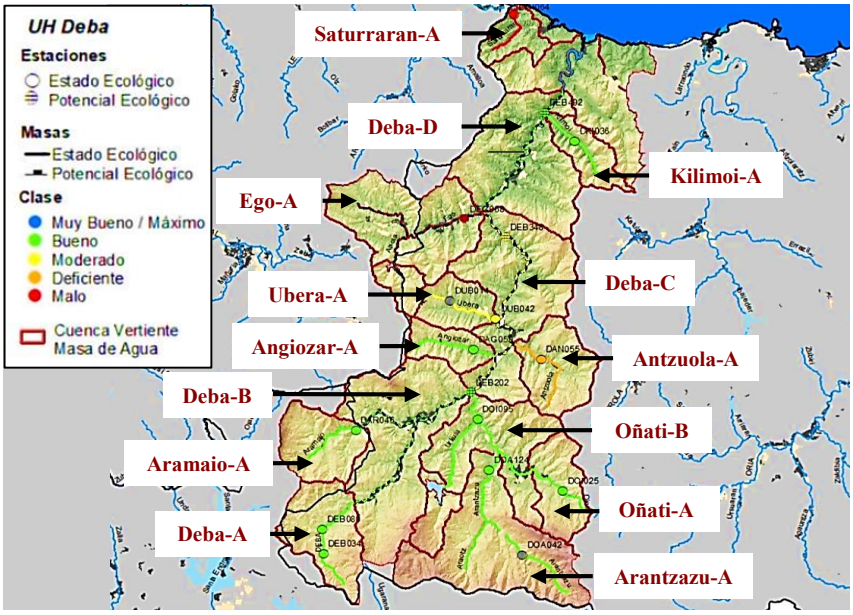

Fig. 2.7. *División de la UH Deba en masas de agua superficial y diagnóstico del estado ecológico para la campaña 2018. Fuente: Figura modificada del informe de la Agencia Vasca del Agua – Ur Agentzia (URA, 2019a)*

Por su parte, el afluente Oñati presentó un buen estado ecológico en todas sus masas durante todo el quinquenio, cumpliendo con el objetivo de buen estado ecológico al 2015.

Por el contrario, el afluente Ego exhibió una calidad fisicoquímica del agua insuficiente e incumplimientos graves y sistemáticos de los indicadores biológicos, no alcanzando todavía el objetivo de buen estado ecológico al 2027.

2.3.1. Red de seguimiento del estado biológico

La red de seguimiento del estado biológico de los ríos (URA, 2019a) consta de la evaluación de los siguientes indicadores biológicos:

a) Composición y abundancia de los organismos fitobentónicos.

b) Composición y abundancia de la fauna bentónica de invertebrados.

c) Composición, abundancia y estructura de la fauna ictiológica (este indicador aún no ha sido validado en el ejercicio de intercalibración por lo que interviene en la determinación del estado o potencial ecológico con un peso inferior al de los organismos fitobentónicos y macroinvertebrados bentónicos).

En el análisis anual de la campaña 2018, se observaron cumplimientos de los estados biológicos para todas las masas de agua, excepto para Deba-C y Ego-A (Tabla 2.6). Durante el quinquenio 2014 – 2018, la masa Deba-A presentó dos incumplimientos debidos a los organismos fitobentónicos en 2015 y 2017, que podrían estar condicionados por las elevadas conductividades eléctricas que presenta este tramo de río a la altura de las salinas de Leintz Gatzaga. En la masa Deba-B, los incumplimientos de la comunidad piscícola fueron sistemáticos, debido principalmente a la escasez de trucha. A su vez, la masa Deba-C también presentó un impacto grave sobre esta comunidad, exhibiendo poca diversidad e incluso ausencia de la trucha. La masa Deba-D presentó incumplimientos ocasionados por macroinvertebrados (especialmente en la campaña 2014), fitobentos o fauna piscícola hasta la campaña 2017. En cuanto a los principales tributarios del río Deba, el afluente Oñati exhibió un excelente estado biológico durante todo el quinquenio, a excepción del incumplimiento de la fauna piscícola en la masa de agua Oñati-B para la campaña 2016. Finalmente, el mal estado biológico del afluente Ego persiste en el tiempo con graves incumplimientos de todos los indicadores biológicos, aunque se aprecia un cambio positivo ligero para la fauna piscícola en las últimas campañas, con registro de la primera trucha en 2018 (Tabla 2.6).

Tabla 2.5. *Evolución de los indicadores del estado o potencial ecológico en la UH Deba en el quinquenio 2014 – 2018. Clasificación de los indicadores: Muy Bueno (MB) en azul, Bueno (B) en verde, Moderado (Mo) en amarillo, Deficiente (D) en naranja y Malo (M) en rojo. Fuente: Tabla modificada del informe de la Agencia Vasca del Agua – Ur Agentzia (URA, 2019a)*

Masa	Indicador	2014	2015	2016	2017	2018
Deba-A	Estado biológico	B	Mo	B	B	B
	Fisicoquímica	B	B	MB	MB	MB
	Hidromorfología	MB	MB	(*)	(*)	(*)
	Estado/potencial ecológico	B	Mo	B	B	B
Deba-B	Estado biológico	Mo	Mo	Mo	B	B
	Fisicoquímica	B	Mo	B	B	B
	Hidromorfología	B	MB	(*)	(*)	(*)
	Estado/potencial ecológico	Mo	Mo	Mo	B	B
Deba-C	Estado biológico	Mo	Mo	Mo	Mo	Mo
	Fisicoquímica	B	B	Mo	B	B
	Hidromorfología	B	B	(*)	(*)	(*)
	Estado/potencial ecológico	Mo	Mo	Mo	Mo	Mo
Deba-D	Estado biológico	M	B	Mo	Mo	B
	Fisicoquímica	Mo	B	B	Mo	B
	Hidromorfología	B	B	(*)	(*)	(*)
	Estado/potencial ecológico	M	B	Mo	Mo	B
Oñati-A	Estado biológico	B	B	B	(*)	B
	Fisicoquímica	B	B	MB	(*)	MB
	Hidromorfología	B	B	(*)	(*)	(*)
	Estado/potencial ecológico	B	B	B	(*)	B
Oñati-B	Estado biológico	B	B	B	B	B
	Fisicoquímica	B	B	MB	B	MB
	Hidromorfología	B	B	(*)	(*)	(*)
	Estado/potencial ecológico	B	B	B	B	B
Ego-A	Estado biológico	M	M	M	M	M
	Fisicoquímica	M	M	D	D	Mo
	Hidromorfología	B	B	(*)	(*)	(*)
	Estado/potencial ecológico	M	M	M	M	M

(*) Indicador no evaluado.

Tabla 2.6. *Evolución de los indicadores del estado biológico en la UH Deba en el quinquenio 2014 – 2018. Clasificación de los indicadores: Muy Bueno (MB) en azul, Bueno (B) en verde, Moderado (Mo) en amarillo, Deficiente (D) en naranja y Malo (M) en rojo. Fuente: Tabla modificada del informe de de la Agencia Vasca del Agua – Ur Agentzia (URA, 2019a)*

Masa	Indicador	2014	2015	2016	2017	2018
Deba-A	Fitobentos	B	Mo	B	Mo	B
	Macroinvertebrados	MB	MB	MB	B	MB
	Fauna piscícola	B	B	B	B	B
	Estado biológico	B	Mo	B	B	B
Deba-B	Fitobentos	Mo	Mo	B	B	MB
	Macroinvertebrados	B	MB	Mo	B	MB
	Fauna piscícola	Mo	Mo	Mo	Mo	Mo
	Estado biológico	Mo	Mo	Mo	B	B
Deba-C	Fitobentos	B	B	B	Mo	Mo
	Macroinvertebrados	MB	MB	MB	MB	MB
	Fauna piscícola	D	D	D	D	D
	Estado biológico	Mo	Mo	Mo	Mo	Mo
Deba-D	Fitobentos	B	B	B	Mo	B
	Macroinvertebrados	M	B	Mo	B	MB
	Fauna piscícola	MB	Mo	Mo	B	B
	Estado biológico	M	B	Mo	Mo	B
Oñati-A	Fitobentos	MB	MB	B	(*)	MB
	Macroinvertebrados	MB	MB	MB	(*)	MB
	Fauna piscícola	B	B	B	(*)	B
	Estado biológico	B	B	B	(*)	B
Oñati-B	Fitobentos	B	MB	B	B	B
	Macroinvertebrados	MB	B	B	B	MB
	Fauna piscícola	B	B	Mo	B	MB
	Estado biológico	B	B	B	B	B
Ego-A	Fitobentos	Mo	D	D	Mo	D
	Macroinvertebrados	M	M	M	M	M
	Fauna piscícola	M	M	Mo	Mo	Mo
	Estado biológico	M	M	M	M	M

(*) Indicador no evaluado.

2.3.2. Red de seguimiento del estado químico

El diseño de la red de seguimiento del estado químico de los ríos (URA, 2019b) permite aportar información para la evaluación de:

i) Los elementos de calidad químicos y fisicoquímicos de soporte a los elementos de calidad biológicos para el cálculo del estado o potencial ecológico: condiciones fisicoquímicas generales (incluyen, condiciones térmicas y de oxigenación, salinidad, estado de acidificación, nutrientes e Índice de Fisicoquímica Referenciado (IFQ-R)) y contaminantes específicos o sustancias preferentes recogidos en el Anexo V del Real Decreto 817/2015.

ii) El estado químico relativo a sustancias prioritarias y otros contaminantes recogidos en el Anexo IV del Real Decreto 817/2015 en las matrices agua, sedimento y biota.

En el análisis anual de la campaña 2018, se observaron cumplimientos de los estados relativos a las condiciones fisicoquímicas generales (CFG) para todas las masas de agua, excepto para Ego-A, debido a incumplimientos por amonio e IFQ-R (Tabla 2.7). Este incumplimiento se produjo en campañas precedentes, ocasionado también por ortofosfatos, DBO_5 y DQO. A excepción del afluente Ego, se observaron buenas condiciones fisicoquímicas de todas las masas de agua durante el quinquenio 2014 – 2018, exceptuando los incumplimientos que se produjeron en la masa Deba-B en 2015 (debido a ortofosfatos y DQO), la masa Deba-C en 2016 (debido al IFQ-R) y la masa Deba-D en 2014 y 2017 (debido al IFQ-R y a ortofosfatos, respectivamente).

De la evaluación del estado fisicoquímico relativo a las sustancias preferentes (Tabla 2.8), se desprende un cumplimiento de los objetivos medioambientales en todas las masas de agua. Por el contrario, en la evaluación del estado químico relativo a las sustancias prioritarias y otros contaminantes (Tabla 2.8) se observan incumplimientos reiterados en las masas Deba-B y Deba-D, esencialmente por superación de la norma de calidad ambiental (NCA) para el mercurio en biota. Los incumplimientos en la masa Ego-A se debieron a superaciones de la NCA para di(2-etilhexil) ftalato (2015) y para clorpirifós (2018) en la matriz agua.

Tabla 2.7. *Evolución de los indicadores del estado fisicoquímico relativo a las condiciones fisicoquímicas generales (CFG) en la UH Deba en el quinquenio 2014 – 2018. Clasificación de los indicadores: Muy Bueno (MB) en azul, Bueno (B) en verde, Moderado (Mo) en amarillo, Deficiente (D) en naranja y Malo (M) en rojo. DBO_5: demanda biológica de oxígeno; DQO: demanda química de oxígeno; IFQ-R: Índice Fisicoquímico Referenciado. Fuente: Elaboración propia a partir de los datos de los informes de la Agencia Vasca del Agua – Ur Agentzia (URA, 2015, 2016, 2017, 2018c y 2019b)*

Masa	Año	CFG	pH	$\%O_2$	NO_3^-	NH_4^+	PO_4^{3-}	DBO_5	DQO	IFQ-R
Deba-A	2014	B	MB	MB	MB	MB	MB	MB	B	MB
	2015	B	MB	MB	MB	MB	MB	B	MB	MB
	2016	MB	MB	MB	MB	MB	MB	MB	MB	MB
	2017	MB	MB	MB	MB	MB	MB	MB	MB	MB
	2018	MB	MB	MB	MB	MB	MB	MB	MB	MB
Deba-B	2014	B	MB	B	MB	B	B	MB	B	B
	2015	Mo	MB	B	B	MB	Mo	B	Mo	B
	2016	B	MB	B	B	MB	MB	B	B	B
	2017	B	MB	B	B	MB	MB	B	B	B
	2018	B	MB	MB	B	MB	MB	MB	B	MB
Deba-C	2014	B	MB	MB	MB	MB	MB	MB	B	MB
	2015	B	MB	B	MB	MB	MB	B	B	B
	2016	Mo	MB	B	MB	MB	MB	B	B	Mo
	2017	B	MB	B	MB	MB	MB	B	MB	MB
	2018	B	MB	B	MB	MB	MB	MB	MB	MB
Deba-D	2014	Mo	MB	B	MB	B	B	MB	B	Mo
	2015	B	MB	MB	MB	MB	B	B	B	B
	2016	B	MB	MB	MB	MB	B	B	B	B
	2017	Mo	MB	MB	MB	MB	Mo	B	B	B
	2018	B	MB	B	MB	MB	MB	B	MB	B
Oñati-A	2014	B	MB	MB	MB	MB	MB	MB	B	MB
	2015	B	MB	MB	MB	MB	MB	B	B	MB
	2016	MB	MB	MB	MB	MB	MB	MB	MB	MB
	2017	(*)	(*)	(*)	(*)	(*)	(*)	(*)	(*)	(*)
	2018	MB	MB	MB	MB	MB	MB	MB	MB	MB
Oñati-B	2014	B	MB	MB	MB	MB	MB	MB	B	MB
	2015	B	MB	B	MB	MB	MB	B	B	MB
	2016	MB	MB	MB	MB	MB	MB	MB	MB	MB
	2017	B	MB	MB	MB	MB	MB	B	MB	MB
	2018	MB	MB	MB	MB	MB	MB	MB	MB	MB
Ego-A	2014	M	MB	B	MB	M	M	D	Mo	M
	2015	M	MB	MB	MB	MB	Mo	Mo	Mo	M
	2016	D	MB	MB	MB	Mo	B	Mo	Mo	D
	2017	D	MB	MB	MB	Mo	Mo	B	B	D
	2018	Mo	MB	MB	MB	Mo	B	B	B	Mo

(*) Indicador no evaluado.

Tabla 2.8. *Evaluación del cumplimiento de objetivos medioambientales relativos a sustancias preferentes (cumplimiento para las categorías Muy Bueno (MB, en azul) y Bueno (B, en verde)) y sustancias prioritarias en la UH Deba para el quinquenio 2014 – 2018. Fuente: Elaboración propia a partir de los datos de los informes de la Agencia Vasca del Agua – Ur Agentzia (URA, 2015, 2016, 2017, 2018c y 2019b)*

	Sustancias Preferentes						Sustancias Prioritarias				
Masa	2014	2015	2016	2017	2018	**Masa**	2014	2015	2016	2017	2018
Deba-A	MB	MB	MB	MB	MB	Deba-A	Cumple	Cumple	Cumple	Cumple	Cumple
Deba-B	MB	MB	MB	MB	MB	Deba-B	Cumple	No cumple	No cumple	No cumple	No cumple
Deba-C	B	MB	MB	MB	MB	Deba-C	Cumple	Cumple	Cumple	Cumple	Cumple
Deba-D	MB	MB	MB	MB	MB	Deba-D	No cumple	No cumple	No cumple	No cumple	No cumple
Oñati-A	MB	MB	MB	(*)	MB	Oñati-A	Cumple	Cumple	Cumple	(*)	Cumple
Oñati-B	MB	MB	MB	MB	MB	Oñati-B	Cumple	Cumple	Cumple	Cumple	Cumple
Ego-A	B	MB	MB	MB	MB	Ego-A	Cumple	No cumple	Cumple	Cumple	No cumple

(*) No evaluado

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3

Objetivos

3.1 Objetivo general

3.2 Objetivos específicos

Fuente: Propia

3. Objetivos

3.1. Objetivo general

La continua intensificación de la industria vasca ha permitido fortalecer el tejido empresarial en el que se sostiene una población en constante crecimiento. Sin embargo, este hecho ha generado múltiples presiones sobre el medio ambiente, llegando a comprometer el estado ecológico de los ríos, a lo largo de los cuales se asientan los mayores núcleos urbanos e industriales. En esta coyuntura se hace imprescindible la consecución de una armonización entre la protección del medio ambiente y un desarrollo regional sostenible. Con este propósito, se han acometido recientemente cambios en la gestión de las aguas residuales urbanas e industriales en la cuenca del río Deba, una de las más contaminadas del País Vasco. A la degradación de la calidad de sus aguas, hay que añadir el impacto que dichos vertidos ocasionan en la calidad de los sedimentos, que actúan como fuente, sumidero y vector de transporte de contaminantes, proporcionando así un registro histórico de la contaminación.

Por tanto, el objetivo principal de este trabajo fue remarcar el papel fundamental de los sedimentos en la dinámica de contaminantes en los ecosistemas acuáticos, y valorar el efecto

de la gestión de vertidos de aguas residuales urbano-industriales sobre el estado ecológico de la cuenca del río Deba, empleando indicadores de calidad fisicoquímica, así como biológica.

3.2. Objetivos específicos

El alcance del objetivo principal se conseguirá a través de los siguientes objetivos parciales:

O.1: Determinar la bioaccesibilidad y distribución química de los metales presentes en los sedimentos superficiales del río con el fin de estimar su potencial para causar efectos perjudiciales sobre la salud humana:

O.1.1: Evaluar la aplicabilidad de un método con base fisiológica que simula las condiciones del tracto gastrointestinal humano para la determinación de la bioaccesibilidad metálica (*Capítulo 5*).

O.1.2: Identificar las principales fuentes de contaminación en la cuenca del río Deba (*Capítulo 5*).

O.2: Evaluar el efecto del tamaño de partícula sobre la geoquímica de los metales en el sedimento y, por ende, sobre el riesgo ecológico y para la salud humana asociados:

O.2.1: Determinar la composición mineralógica, elemental y metálica para la caracterización de dos tamaños de partícula distintos −fracción fina (< 63 μm) y gruesa (< 2 mm)− del sedimento superficial (*Capítulo 6*).

O.2.2: Evaluar la influencia de la estacionalidad de las condiciones hidrológicas del río sobre la migración de los metales asociados a las partículas en suspensión hacia la zona litoral (*Capítulo 6*).

O.3: Evaluar el potencial bioindicador de la actividad y biodiversidad microbiana involucrada en el ciclo del nitrógeno en los sedimentos para determinar el estado ecológico de la cuenca del río Deba:

O.3.1: Determinar, a escala de laboratorio mediante la adaptación de técnicas

de medida de actividades enzimáticas en plantas y cultivos de bacterias aisladas, la actividad de la Nitrato Reductasa y la Nitrito Reductasa en los sedimentos (*Capítulo 7*).

O.3.2: Analizar la biodiversidad (funcional y estructural) de las comunidades bacterianas presentes en los sedimentos mediante la combinación de dos técnicas de biología molecular: reacción en cadena de la polimerasa cuantitativa (en inglés, quantitative polymerase chain reaction (qPCR)) y metabarcoding (*Capítulo 8*).

O.3.3: Identificar posibles alteraciones de la actividad y biodiversidad microbiana en el sedimento para evaluar el impacto de las distintas fuentes de contaminación sobre el estado ecológico de la cuenca del río Deba (*Capítulo 7 y Capítulo 8*).

O.4: Desarrollar un índice multimétrico, tanto para el agua como el sedimento, que vincule parámetros químicos con biológicos para inferir un nuevo sistema de control del estado ecológico de una cuenca (*Capítulo 9*).

Source: Own source

3. Objectives

3.1. General objective

Continuous intensification of Basque industry has allowed to strengthen the business fabric, which sustains a constant growing population. Nevertheless, this fact has generated multiple pressures on the environment and, consequently, it compromises the ecological status of rivers, where the most important urban and industrial areas are concentrated. In this situation, balancing the environmental protection with a sustainable development of the region becomes an essential goal. With this purpose, recent changes have been made in the management of urban and industrial wastewaters in the Deba River, one of the most polluted catchments in the Basque Country. In addition to the degradation of water quality, the impact of wastewater discharges on sediment quality should be considered. Indeed, sediment acts as source, sink and transport vector of pollutants, providing a history of contamination.

Therefore, the main aim of this work was to emphasize the key role of sediments in pollutant dynamics in aquatic ecosystems, and to evaluate the influence of urban and industrial wastewaters management on the ecological status of the Deba River catchment, using both physicochemical and biological quality indicators.

3.2. Specific objectives

The main objective will be achieved through the following specific objectives:

O.1: To determine the bioaccessibility and chemical distribution of metals in river surface sediments for estimating the potential for metals to cause harmful effects in exposed people.

O.1.1: To evaluate whether a physiologically based method for human gastrointestinal environment simulation is applicable for determining metal bioaccessibility (*Chapter 5*).

O.1.2: To identify the main contamination sources in the Deba River catchment (*Chapter 5*).

O.2: To evaluate the effect of particle size on geochemistry of metals in sediment and, therefore, on the associated ecological and human health risk:

O.2.1: To determine the mineralogical, elemental and metal composition to characterise two different particle sizes −fine (< 63 μm) and bulk (< 2 mm) fractions− of sediments (*Chapter 6*).

O.2.2: To evaluate the influence of river hydrological conditions seasonality on metal migration towards the coastal area (*Chapter 6*).

O.2.3: To evaluate the potential for the activity and biodiversity of microbial community involved in nitrogen cycle in sediments to determine the ecological status of the Deba River catchment.

O.3: To determine, on laboratory scale, Nitrate Reductase and Nitrite Reductase activities in sediments by adapting techniques for the measurement of enzymatic activities in plants and isolated bacteria cultures (*Chapter 7*).

O.3.1: To analyze the structure and function of bacterial communities present in sediments by combining two molecular biological techniques: quantitative

polymerase chain reaction (qPCR) and metabarcoding (*Chapter 8*).

O.3.2: To identify disruptions in microbial activity and biodiversity in sediments for evaluating the impact of different contamination sources on the ecological status of the Deba River catchment (*Chapter 7 and Chapter 8*).

O.4: To develop a multimetric index for both water and sediments, linking chemical with biological data for inferring a new control system for monitoring the ecological status of a catchment (*Chapter 9*).

4

Materiales y métodos

Fuente: Propia

4. Materiales y métodos

4.1. Recolección y acondicionamiento de muestras

Siguiendo el protocolo establecido por la Agencia de Protección Ambiental de Estados Unidos (USEPA, 2001), las submuestras de sedimento superficial (entre 0 y 5 cm de profundidad), procedentes de múltiples puntos de todo el ancho del cauce del río, se recolectaron con la ayuda de una cuchara de plástico y se tamizaron a través de una malla de 2 mm de abertura. A continuación, y tras una correcta homogeneización, las porciones de muestra compuesta se depositaron en contenedores de polipropileno. Por su parte, las muestras de agua se recolectaron en botes de polietileno.

Todo el instrumental empleado en el muestreo fue previamente esterilizado y las muestras, tanto de agua como de sedimento, se transportaron perfectamente precintadas, refrigeradas y en la oscuridad para evitar posibles alteraciones durante su transporte al laboratorio.

Una vez en el laboratorio, las muestras de sedimento se acondicionaron de las siguientes tres maneras, dependiendo del análisis al que fueran a someterse:

i) Aquellas muestras destinadas al análisis físicoquímico y determinación de actividades enzimáticas (A), se secaron al aire, se homogeneizaron con la ayuda de un mortero y se almacenaron a 4°C hasta su análisis. Para la obtención de la fracción fina (< 63 μm) de sedimento (considerada en los Capítulos 5 y 6), las muestras se tamizaron a través de una malla de 63 μm de abertura.

ii) Aquellas muestras destinadas únicamente a los ensayos 1 (establecimiento del protocolo para acondicionamiento de muestras de sedimento y extracción de enzimas previa a la determinación de actividades enzimáticas) y 2 (estudio de la cinética de las actividades enzimáticas) (B), posteriormente descritos en los apartados 4.2.1 y 4.2.2, respectivamente, se almacenaron frescas a 4°C hasta su análisis.

iii) Aquellas muestras destinadas a la extracción de ADN (en inglés, DNA) (C), se almacenaron frescas a -20°C.

Por su parte, las muestras de agua se acondicionaron de las siguientes dos maneras:

i) Una muestra (A) se almacenó bruta a 4°C.

ii) Otra muestra se filtró a través de un filtro de nitrocelulosa con un tamaño de poro de 0,45 μm. Una réplica (B) de la muestra de agua filtrada se almacenó directamente a 4°C hasta su análisis, mientras que la otra (C) se acidificó previamente con HNO_3 concentrado (69%).

4.2. Descripción de los ensayos

4.2.1. Ensayo 1: establecimiento del protocolo para acondicionamiento de muestras de sedimento y extracción de enzimas previa a la determinación de actividades enzimáticas (Capítulo 7)

El objetivo del presente ensayo era establecer un protocolo adecuado para la cuantificación, a escala laboratorio, de la actividad de dos enzimas (Nitrato Reductasa y Nitrito Reductasa) involucradas en la desnitrificación bacteriana en muestras de sedimento superficial, con el fin de evaluar su potencial bioindicador del estado ecológico de la cuenca del río Deba.

Tras una intensa revisión bibliográfica, se constató que una gran mayoría de estudios centrados en la determinación de actividades enzimáticas en suelos o sedimentos, se basaban en la cuantificación colorimétrica del producto liberado por la actividad de la enzima objeto de estudio tras la adición de un sustrato específico. Por su parte, las técnicas de determinación de actividades enzimáticas en plantas y/o cultivos de bacterias aisladas, ampliamente extendidas, incluían una etapa adicional que consistía en la extracción previa de las enzimas ya que son, en su mayoría, intracelulares. Concretamente, la enzima Nitrato Reductasa procariota, capaz de catalizar la reducción de nitratos a nitritos durante la desnitrificación, se localiza en el periplasma o asociada a la membrana. A su vez, la enzima Nitrito Reductasa procariota, capaz de catalizar la reducción de nitritos a monóxido de nitrógeno durante la desnitrificación, únicamente se encuentra en el periplasma (Moreno-Vivián et al., 1999).

Por consiguiente, se optó por establecer un protocolo para (1) el acondicionamiento de muestras de sedimento y (2) la extracción de enzimas previa a la determinación de las actividades enzimáticas.

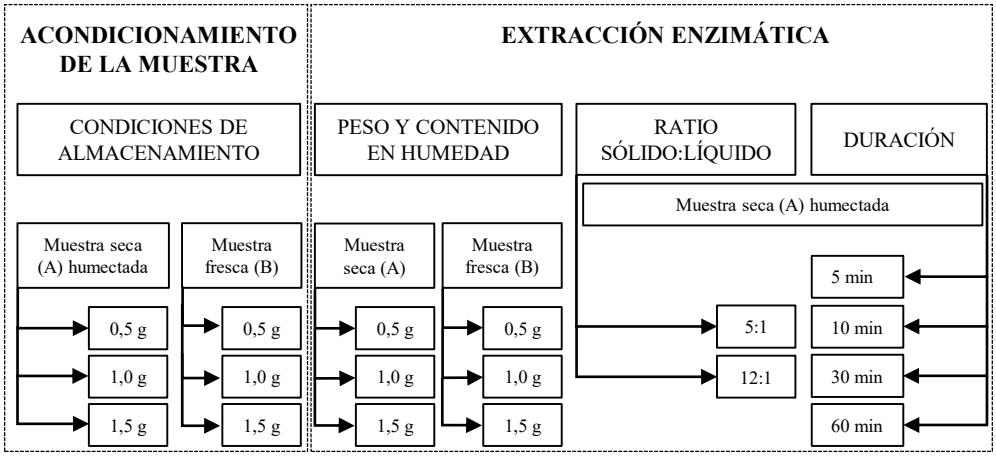

Fig. 4.1. *Esquema del procedimiento seguido durante el Ensayo 1*

Inicialmente, se dispusieron en bandejas de plástico dos muestras de sedimento superficial: una seca (A) que se humectó con agua Milli-Q hasta un contenido de humedad del 30%, y otra fresca (B), ambas almacenadas en el laboratorio a 4°C hasta su análisis, tal y como se describe en el apartado 4.1. Estas muestras se incubaron a 13°C (temperatura media

del agua del río) durante dos días para la aclimatación de las bacterias.

Puesto que existían evidencias que sugerían que la Nitrato Reductasa es más sensible a la inactivación durante la extracción que la Nitrito Reductasa (Buczek, 1984), para el propósito de este ensayo se decidió determinar la actividad Nitrato Reductasa. Para ello, se siguió la metodología empleada por Antolín et al., (2010).

Inicialmente, se dispusieron en recipientes de polipropileno tres réplicas para cada uno de los pesos seleccionados (0,5, 1,0 y 1,5 g) correspondientes a (1) las muestras seca (A) humectada y fresca (B) previamente incubadas; y (2) muestras seca (A) y fresca (B) sin incubación previa (Fig. 4.1). A cada réplica se adicionaron 5 mL de una disolución extractante de enzimas que consistía en una solución tampón fosfato potásico 50 mM (pH = 7.5) + EDTA 2 mM + DTT 2 mM + PVPP insoluble (1%, m/v) + caseína soluble (1,5%, m/v) a 0°C, y la mezcla se centrifugó a 4300 r.p.m durante 5 minutos. Seguidamente, se tomaron por triplicado 0,5 mL de sobrenadante, al que se agregaron 0,1 mL de solución tampón fosfato potásico 50 mM (pH = 7,5), 0,1 mL de una disolución de NADH (1 mg L^{-1}), 0,2 mL de una disolución de KNO_3 100 mM y agua Milli-Q hasta un volumen final de 2 mL. Tras 15 minutos de incubación a 28°C en la oscuridad, se tomó una alícuota de 0,8 mL y se diluyó con agua Milli-Q hasta un volumen final de 10 mL. El contenido de NO_2^- en la alícuota y posterior cuantificación de la actividad se realizó tal y como se describe en el apartado 4.3.2.1.

Paralelamente, se estudió el efecto de (1) adicionar distintos volúmenes (5 y 12 mL) de disolución extractante a 1,0 g de muestra seca (A) humectada e incubada previamente a 13°C durante dos días, y de (2) prolongar la extracción enzimática durante 5, 10, 30 y 60 minutos (Fig. 4.1).

4.2.2. Ensayo 2: estudio de la cinética de las actividades enzimáticas (Capítulo 7)

Como continuación del ensayo 1, cuyo objetivo era establecer un protocolo adecuado para la cuantificación, a escala laboratorio, de la actividad Nitrato Reductasa y Nitrito Reductasa en muestras de sedimento superficial, el presente ensayo se centró en el estudio de la cinética de ambas actividades enzimáticas. Además, se evaluó el efecto de adicionar

distintas cantidades de sustrato (KNO_3 y KNO_2, respectivamente) al medio de incubación.

Tal y como se observa en la Fig. 4.2, se dispusieron en tubos de ensayo tres réplicas de extracto crudo para cada una de las concentraciones de disolución de KNO_3 (0, 1, 10 y 100 mM) adicionada y para cada tiempo de incubación (0, 15, 30, 45, 60, 75, 90, 105 y 120 minutos) en la determinación de la actividad Nitrato Reductasa. De la misma manera, se dispusieron en tubos de ensayo tres réplicas de extracto crudo para cada una de las concentraciones de disolución de KNO_2 (0 y 2,5 mM) adicionada y para cada tiempo de incubación (0, 15, 30, 45, 60, 75, 90, 105 y 120 minutos) en la determinación de la actividad Nitrito Reductasa.

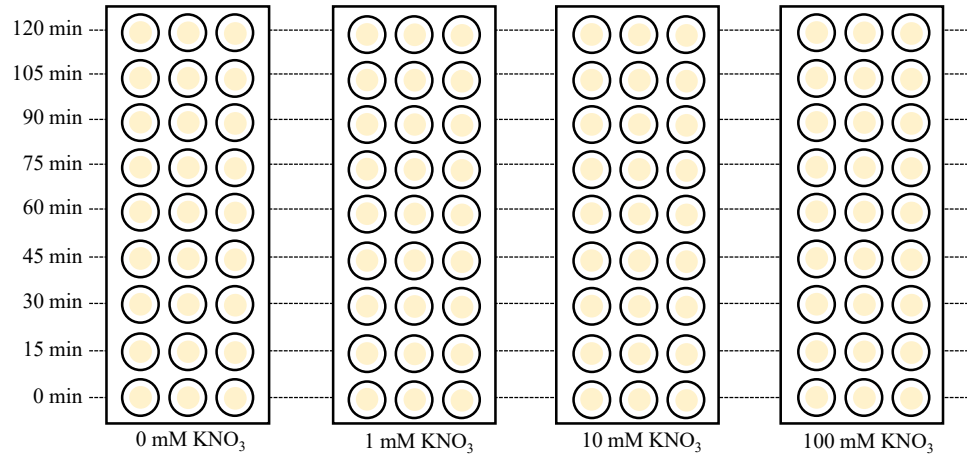

Fig. 4.2. *Esquema del procedimiento seguido durante el Ensayo 2*

4.2.3. Ensayo 3: efecto de la humectación prolongada sobre las actividades enzimáticas (Capítulo 7)

Una vez validada la metodología experimental, uno de los aspectos más interesantes del presente ensayo fue el estudio del efecto de la humectación prolongada de las muestras de sedimento superficial sobre la evolución tanto de las actividades enzimáticas como del contenido de nutrientes (NH_4^+ y NO_3^-). Para ello, se dispusieron en bandejas de plástico muestras de sedimento superficial secas (A) que se humectaron inicialmente con agua Milli-Q hasta un contenido de humedad del 30%, y se incubaron a 13°C (temperatura media del agua del río) durante cinco días. Cada día se tomaron muestras de sedimento para determinar el

contenido de humedad, NH_4^+ y NO_3^-, así como las actividades Nitrato Reductasa y Nitrito Reductasa. Además, la humedad de las muestras se mantuvo constante añadiendo agua Milli-Q cuando fuese necesario.

4.3. Métodos analíticos

4.3.1. Parámetros fisicoquímicos

Dentro de estos parámetros se incluyen todas aquellas determinaciones analíticas realizadas para determinar la composición fisicoquímica de las muestras de agua y sedimento superficial procedentes del río. Todas ellas se realizaron por triplicado.

4.3.1.1. Muestra de agua

4.3.1.1.1 Muestra de agua bruta

A continuación, se detallan las determinaciones analíticas realizadas en las muestras de agua bruta (A).

La **determinación del pH, la conductividad eléctrica** (en inglés, electrical conductivity (**EC**)) **y el potencial redox (Eh)** del agua se realizó *in situ* en el río, empleándose un medidor Micro pH 2000 (Crison), un conductímetro Basic 30+ (Crison) y una sonda ORP/Redox serie MTC101 (Hach) con electrodo de Ag/AgCl, respectivamente.

La **determinación del contenido de carbono orgánico particulado** (en inglés, particulate organic carbon (**POC**)) en el agua se realizó de acuerdo con el método 5301B (APHA, 2015) empleando un analizador de carbono orgánico total (TOC-L Shimadzu).

La determinación del **contenido de materia particulada en suspensión (**en inglés, suspended particulate matter (**SPM**)) en el agua se realizó a partir de la filtración de la muestra bruta (A) para la obtención de la muestra filtrada (B y C). Tras secar el residuo retenido en el filtro en una estufa a 105°C hasta peso constante (1 h aproximadamente), el contenido de materia particulada en suspensión en el agua (m/v) se calculó como cociente entre el peso del residuo y el volumen de muestra filtrada (APHA, 2015).

La determinación del **contenido pseudo-total de metales particulados** (Fe, Mn, Zn, Ni, Cu, Cr y Pb) en el agua, se realizó empleando un sistema de digestión por microondas (ETHOS 1, Millestone), en el que se digirió el residuo retenido en el filtro y dispuesto en un tubo de teflón, con una combinación de 3 mL de HNO_3 concentrado (65%), 1,5 mL de $HClO_4$ concentrado (60%) y 10,5 mL de agua Milli-Q. El programa de digestión consistía en una etapa de 10 minutos de aumento de la temperatura hasta los 180°C y una etapa de 25 minutos adicionales a temperatura (180°C) constante (USEPA, 2007b). Tras la digestión, el contenido del tubo de teflón se filtró a través de un filtro de nitrocelulosa (Millipore) con un tamaño de poro de 0,45 µm, se adicionó agua Milli-Q hasta completar un volumen total de 50 mL y volvió a filtrase con un filtro de nitrocelulosa (Millipore) con un tamaño de poro de 0,45 µm acoplado a una jeringa. Por último, el contenido pseudo-total de metales en el producto de la digestión se determinó mediante un espectrómetro de emisión óptica con acoplamiento de plasma inductivo (en inglés: inductively coupled plasma - optical emission spectrometer (ICP-OES)) (Perkin Elmer 2000). Por tanto, el contenido pseudo-total de metales particulados (m/m) se calculó como cociente entre la concentración pseudo-total de los metales en la digestión del residuo retenido en el filtro (m/v) y la concentración de materia particulada en suspensión en el volumen de agua bruta (m/v). Los límites de detección para estos metales fueron: Pb (1 µg g^{-1}), Zn (0,5 µg g^{-1}), Fe y Mn (0,4 µg g^{-1}), y Cr, Cu y Ni (0,1 µg g^{-1}).

4.3.1.1.2 Muestra de agua filtrada sin acidificar

A continuación, se detallan las determinaciones analíticas realizadas en las muestras de agua filtrada sin acidificar (B).

La **determinación del contenido de carbono orgánico disuelto** (en inglés, dissolved organic carbon (**DOC**)) en el agua, se realizó de acuerdo con el método 5301B (APHA, 2015) empleando un analizador de carbono orgánico total (TOC-L Shimadzu).

La **determinación del contenido de aniones solubles** (NO_3^-, SO_4^{2-} y Cl^-) en el agua, se realizó empleando un cromatógrafo iónico (DIONEX ICS 3000).

4.3.1.1.3 Muestra de agua filtrada y acidificada

A continuación, se detallan las determinaciones analíticas realizadas en las muestras de agua filtradas y posteriormente acidificadas (C).

La **determinación del contenido de cationes solubles** (Ca^{2+}, Mg^{2+}, Na^{+} y K^{+}) en el agua se realizó mediante espectrometría de emisión óptica con acoplamiento de plasma inductivo. Los límites de detección para estos elementos fueron: Ca (0,5 µg L^{-1}), Mg (µg L^{-1}), Na (5 µg L^{-1}) y K (10 µg L^{-1}).

La **determinación del contenido de NH_4^+** en el agua se realizó llevando a cabo la reacción de Berthelot modificada (Krom, 1980). A 2 mL de muestra se adicionaron 1 mL de una disolución (1:1:1) de NaOH 0,3 M, salicilato sódico (17%, m/v) + nitroprusiato sódico (0,12% m/v) y agua Milli-Q, y 0,5 mL de otra disolución de dicloroisocianurato (0,1%, m/v). Tras 30 minutos de reacción se midió la absorbancia (λ = 670 nm) mediante un espectrofotómetro ultravioleta (Jasco V-630).

La **determinación del contenido de NO_2^-** en el agua se realizó siguiendo el método 354.1 (USEPA, 1979). A 50 mL de muestra se adicionaron 2 mL de una disolución de HCl (7,7% v/v), acetato sódico (27,2%, m/v), sulfanilamida (1%, m/v) y N-(1-naftil)-etilendiamina diclorhidrato (0,1%, m/v). Tras 15 min de reacción se midió la absorbancia (λ = 540 nm) mediante espectrometría ultravioleta.

La **determinación del contenido de PO_4^{3-}** en el agua se realizó siguiendo el método 4500-P E (APHA, 2015). Previamente, a 5 mL de muestra se adicionaron 10 µL de una disolución de fenolftaleína (0,1%, m/v) en etanol. Tras el viraje del color a violeta se adicionaron repetidamente 10 µL de una disolución de H_2SO_4 5N hasta su neutralización. Posteriormente, se adicionaron 0,75 mL de una disolución que contenía 50 mL de H_2SO_4 5N, 5 mL de tartrato antimónico potásico (0,27%, m/v), 15 mL de molibdato de amonio (4%, m/v) y 30 mL de ácido ascórbico 0,1M. Tras 10-30 minutos de reacción se midió la absorbancia (λ = 880 nm) mediante espectrometría ultravioleta.

La **determinación del contenido de metales solubles** (Fe, Mn, Zn, Ni, Cu, Cr y Pb) en el agua se realizó empleando un espectrómetro de emisión óptica con acoplamiento de

plasma inductivo y un nebulizador ultrasónico (CETAC, U5000AT). El límite de detección para estos metales fue de 0,5 µg L^{-1}.

4.3.1.2. Muestras de sedimento superficial

A continuación, se detallan las determinaciones analíticas realizadas en las muestras de sedimento secadas al aire (A).

Para la determinación del **contenido de humedad** en el sedimento, se secaron 10 g de muestra en una estufa a 105°C hasta peso constante. El contenido en humedad (%) se calculó a partir de la diferencia de peso antes y después del secado (APHA, 2015).

Para la **caracterización molecular/mineralógica** del sedimento, se utilizaron técnicas espectroscópicas como la difracción de rayos X (en inglés, X-ray Diffraction (XRD)) e infrarrojo (en inglés, Infrared (IR)). Los análisis de XRD se realizaron utilizando un difractómetro para muestras en polvo (Xpert PRO PANalytical) operando a 40 k y 40 mA, equipado con un tubo de cobre (λCuKαmedia = 1,5418 Å, λCuKα1 = 1,54060 Å, λCuKα2 = 1,54439 Å) y un detector PixCel. El análisis semicuantitativo de los compuestos presentes en cada muestra de sedimento se realizó con ayuda del software PANalytical X'pert HighScore. La espectroscopia de IR se llevó a cabo mediante un espectrofotómetro de IR (Jasco 6300 FTIR) en modo de transmisión. Todos los espectros de IR obtenidos se recogieron en la región del infrarrojo medio (de 4000 a 400 cm^{-1}), con una resolución espectral de 4 cm^{-1}.

Para la **caracterización elemental** del sedimento se utilizó una técnica combinada de microscopía electrónica de barrido con espectrometría de dispersión de energía de Rayos X (en inglés, scanning electron microscopy with energy dispersive spectroscopy (SEM-EDS)). Inicialmente, se prensaron 0,5 g de muestra que fue sometida a una presión de 9 t en una prensa hidráulica CrushIR (PIKE Technologies) hasta obtener una pastilla de aproximadamente 12 mm de diámetro y 1 mm de grosor. Posteriormente, las muestras en pellets fueron analizadas mediante X-Max EDS (Oxford Instruments) acoplado a un microscopio electrónico de barrido EVO40 (Carl Zeiss NTS GmbH). Las imágenes SEM se obtuvieron a alto vacío, empleando un voltaje de aceleración de 20 KV. Además, un detector de electrones secundarios permitió magnificaciones de 10.000 x. Finalmente, el análisis elemental se llevó a cabo utilizando una

distancia de trabajo de 8,5 mm, un ángulo de incidencia de 35° y una tensión de aceleración de 30 kV. Además, se utilizó un tiempo de integración de 50 s para mejorar la relación señal-ruido de los espectros EDS. Los datos espectrales se procesaron utilizando el software INCA (Oxford Instruments), que proporcionó una aproximación semicuantitativa de los elementos contenidos en las muestras de sedimento en función de las áreas netas de K-alfa de cada elemento detectado. La información adicional relacionada con la instrumentación, las condiciones de medición y la asignación espectral se puede revisar en Aramendia et al., (2018) y Gómez-Nubla et al., (2013).

Para la determinación del **contenido total de carbono, nitrógeno y azufre** en el sedimento, se empleó un analizador elemental TruSpec CHNS (Leco Corporation) en el que se introdujeron entre 0,1 y 0,5 g de muestra.

Para la determinación del **contenido total de carbono orgánico** (en ingles, total organic carbon (**TOC**)) del sedimento se aplicó el método normalizado 2540 E para la determinación de sólidos volátiles (APHA, 2015). Se calcinaron 5 g de muestra (previamente secada en estufa a 105°C) en una mufla a 550°C hasta peso constante. El porcentaje de peso perdido tras la calcinación se consideró representativo del contenido total de carbono orgánico.

Para la determinación del **contenido de NH_4^+ y NO_3^-** del sedimento, se realizó una extracción con KCl 1M (adaptado de Mulvaney, 1996). Tras someter a agitación 10 g de muestra con 20 mL de una disolución KCl 1M durante 1h a 170 r.p.m, se centrifugó a 4500 r.p.m durante 5 minutos y el sobrenadante se filtró con un filtro de nitrocelulosa (Millipore) con un tamaño de poro de 0,45 µm acoplado a una jeringa. Para determinar el contenido de NH_4^+ en el sobrenadante se aplicó el procedimiento descrito para la determinación de amonio en el agua (Apartado 4.3.1.1.3). A su vez, para determinar el contenido de NO_3^- se clarificaron 2 mL de sobrenadante con 5 mL de una disolución de crema de alúmina (0,46%, m/v) y se centrifugaron a 4500 r.p.m durante 5 minutos. Finalmente, se midió la absorbancia (λ = 220 nm) mediante espectrometría ultravioleta, eliminando la absorbancia debida a compuestos orgánicos (λ = 275 nm).

Para la determinación del **contenido pseudo-total de metales** (Fe, Mn, Zn, Ni, Cu, Cr y Pb) en el sedimento, se digirieron 0,5 g de muestra siguiendo el mismo procedimiento

descrito previamente para la determinación del contenido pseudo-total de metales particulados en el agua (Apartado 4.3.1.1.1). Tras la digestión, el contenido del tubo de teflón se filtró a través de un filtro de nitrocelulosa (Millipore) con un tamaño de poro de 0,45 µm, se adicionó agua Milli-Q hasta completar un volumen total de 100 mL y volvió a filtrase con un filtro de nitrocelulosa (Millipore) con un tamaño de poro de 0,45 µm acoplado a una jeringa. Finalmente, el contenido pseudo-total de metales se determinó mediante espectrometría de emisión óptica con acoplamiento de plasma inductivo. Para el control de esta técnica analítica, se analizó adicionalmente una muestra de sedimento certificada NBS (Río Buffalo, EEUU), obteniéndose contenidos medios cercanos a los valores certificados con desviaciones inferiores al 8% para todos los metales, excepto para el Pb (17% de desviación al analizar 0,5 g de muestra).

Para la determinación de la **distribución química de metales** (Fe, Mn, Zn, Ni, Cu, Cr y Pb) en el sedimento, se aplicó un protocolo de extracción secuencial basado en el procedimiento desarrollado por Tessier et al., (1979) y modificado por Baffi et al., (1998), con mejoras establecidas de conformidad con la Oficina Comunitaria de Referencia Europea (en inglés: European Community Bureau of Reference (BCR 701)). Esta metodología está dividida en cuatro etapas operacionales:

1) Fracción intercambiable, extraíble con agua y ácido (F_1; extracción de metales asociados a especies solubles, carbonatos y en forma de iones intercambiables): se mezclaron en un recipiente de polietileno 0,5 g de muestra y 20 mL de una disolución de CH3COOH 0,11 M. Posteriormente, el recipiente se tapó y se dispuso en un baño agitador horizontal a 30 r.p.m durante 16 h a 20-22°C.

2) Fracción reducible (F_2; extracción de metales asociados a oxihidróxidos de Fe y Mn): al residuo de la primera etapa (F_1) se le adicionaron 20 mL de una disolución de $NH_2OH{\cdot}HCl$ 0,5 M acidificada con HNO_3 concentrado (69%) hasta pH = 2. El contenido volvió a someterse a agitación horizontal a 30 r.p.m durante 16 h a 20-22°C.

3) Fracción oxidable (F_3; extracción de metales asociados a materia orgánica y sulfuros): inicialmente, al residuo de la segunda etapa (F_2) se le adicionaron 10 mL de H_2O_2 concentrado (30% m/v) y se dispuso nuevamente en el baño agitador a 30

r.p.m durante 1 h a 85°C (este paso se realizó por duplicado y al finalizar el recipiente se destapó hasta el secado completo del residuo). Posteriormente, y una vez enfriada la muestra, se adicionaron 25 mL de una disolución de NH_4CH_3COO 1M acidificada con HNO_3 concentrado (69%) hasta pH = 2, y se volvió a agitar la mezcla a 30 r.p.m durante 16 h a 20-22°C.

4) Fracción residual (F_4: determinación de los metales remanentes asociados a la estructura cristalina del sedimento): el residuo de la tercera etapa (F_3) se trasvasó completamente a un tubo de teflón y se digirió con una combinación 3 mL de HNO_3 concentrado (65%), 1,5 mL de $HClO_4$ concentrado (70%) y 10,5 mL de agua Mili-Q, siguiendo el procedimiento descrito anteriormente para la determinación del contenido pseudo-total de metales en el sedimento.

Al finalizar cada una de las etapas de extracción, la mezcla producto final del procedimiento se sometió a centrifugación a 3000 r.p.m. durante 40 min. El líquido sobrenadante se filtró a través de un filtro de nitrocelulosa (Millipore) con un tamaño de poro de 0,45 µm acoplado a una jeringa, depositándolo en tubos de polietileno almacenados a 4°C hasta el análisis. La determinación del contenido de metales en cada extracto se realizó mediante espectrometría de emisión óptica con acoplamiento de plasma inductivo. De esta manera, el porcentaje de metal asociado a cada fracción del sedimento se calculó como cociente entre el contenido de metal en el extracto y la suma del contenido de metal en las cuatro fracciones.

4.3.1.3. Análisis de la bioaccesibilidad de los metales en el sedimento

Tras una intensa revisión bibliográfica, se constató la gran diversidad de metodologías existentes para el análisis, a escala laboratorio, de la bioaccesibilidad de los metales en el sedimento (ISO 17402:2008). Estos procedimientos se clasifican en dos grandes grupos: químicos y fisiológicos. Mientras los primeros utilizan exclusivamente reactivos químicos sin ningún acondicionamiento fisiológico, los segundos incluyen el empleo de reactivos más complejos que se corresponden con análogos gástricos e intestinales (e.g., ácidos orgánicos, enzimas o sales biliares).

Con el objetivo de comprender la necesidad de reproducir con fidelidad el tracto gastrointestinal para estimar el riesgo para la salud humana asociado a la ingesta de sedimentos contaminados, se optó por seguir dos metodologías: una química, el Procedimiento de Lixiviación Característica de la Toxicidad (en inglés, Toxicity Characteristic Leaching Procedure (TCLP)) y otra fisiológica, la Prueba de Extracción con Base Fisiológica (en inglés, Physiologically Based Extraction Test (PBET)).

La determinación de la **bioaccesibilidad de los metales** (Fe, Mn, Zn, Ni, Cu, Cr y Pb) en el sedimento de acuerdo con la prueba química **TCLP**, se llevó a cabo de conformidad con el método normalizado 1311 (USEPA, 1993). Se mezclaron en un recipiente de polietileno 1 g de muestra y 20 mL de una disolución de CH_3COOH 0,1 M. Posteriormente, el recipiente se tapó y se dispuso en un baño agitador horizontal a 30 r.p.m durante 18 h a 20-22°C. Tras la extracción, la mezcla producto final del procedimiento se sometió a centrifugación a 3000 r.p.m. durante 20 min. El líquido sobrenadante se filtró a través de un filtro de nitrocelulosa (Millipore) con un tamaño de poro de 0,45 µm acoplado a una jeringa, depositándolo en tubos de polietileno. Finalmente, se adicionaron 40 µL de HNO_3 concentrado (69%) para ajustar el pH por debajo de 2 y se almacenó a 4°C hasta su análisis.

El contenido de metales en el extracto se determinó mediante espectrometría de emisión óptica con acoplamiento de plasma inductivo. De esta manera, la bioaccesibilidad (%) se calculó como el cociente entre el contenido de metal en el extracto y el contenido pseudo-total de metal en la muestra de sedimento.

La determinación de la **bioaccesibilidad de los metales** (Fe, Mn, Zn, Ni, Cu, Cr y Pb) en el sedimento de acuerdo con la prueba fisiológica **PBET**, se llevó a cabo siguiendo el procedimiento diseñado por Ruby et al., (1993) y modificado por Drexler, cuyas mejoras se incluyeron en el método normalizado establecido por la Agencia de Protección del Medio Ambiente de Estados Unidos (USEPA, 2007a). Esta metodología está dividida en dos etapas operacionales:

1) Fase gástrica (simulación de las condiciones del compartimento estomacal): inicialmente, se preparó la disolución gástrica, que contenía 0,625 g de pepsina, 210 µL de ácido láctico y 250 µL de ácido acético glacial, diluidos con agua Milli-Q hasta

completar un volumen de 500 mL. El pH de esta disolución se ajustó a 2,5 con la adición gota a gota de HCl concentrado (37%). A continuación, se mezclaron en un recipiente de polietileno 0,5 g de muestra y 50 mL de la disolución gástrica. Tras tapar el recipiente se dispuso en un baño agitador horizontal a 60 r.p.m durante 1h a 37°C. Posteriormente, se extrajo una alícuota de 20 mL y se centrifugó a 2000 r.p.m durante 20 min. El líquido sobrenadante se filtró a través de un filtro de nitrocelulosa (Millipore) con un tamaño de poro de 0,45 µm acoplado a una jeringa, depositándolo en tubos de polietileno. Finalmente, se adicionaron 50 µL de HNO_3 concentrado (69%) para ajustar el pH por debajo de 2 y se almacenó a 4°C para su conservación hasta el análisis.

2) Fase intestinal (simulación de las condiciones del compartimento intestinal): al residuo de la fase gástrica se le añadieron 20 mL adicionales de disolución gástrica, 70 mg de sales biliares, 20 mg de pancreatina y unas gotas de $NaHCO_3$ 1M hasta ajustar el pH a 7. Tras tapar el recipiente, se dispuso nuevamente en un baño agitador horizontal a 60 r.p.m durante 3h a 37°C. Posteriormente, se extrajo una alícuota de 20 mL y se centrifugó a 2000 r.p.m durante 20 min. El líquido sobrenadante se filtró a través de un filtro de papel (Whatman número 2) y, a continuación, a través de un filtro de nitrocelulosa (Millipore) con un tamaño de poro de 0,45 µm acoplado a una jeringa, depositándolo en tubos de polietileno. Finalmente, se adicionaron 120 µL de HNO_3 concentrado (69%) para ajustar el pH por debajo de 2 y se almacenó a 4°C para su conservación hasta el análisis.

La determinación del contenido de metales en ambos extractos se realizó mediante espectrometría de emisión óptica con acoplamiento de plasma inductivo. De esta manera, la bioaccesibilidad (%) correspondiente a cada fase se calculó como el cociente entre el contenido de metal en el extracto y el contenido pseudo-total de metal en la muestra de sedimento.

4.3.2. Parámetros microbiológicos

Dentro de estos parámetros se incluyen todas aquellas determinaciones analíticas realizadas por triplicado para determinar la actividad y biodiversidad (funcional y estructural) microbiana en las muestras de sedimento superficial procedentes del río.

4.3.2.1. Bioindicadores específicos del ciclo del nitrógeno

Los protocolos establecidos para la determinación de actividades enzimáticas involucradas en la desnitrificación bacteriana en sedimentos se basan en técnicas de medida en plantas y/o cultivos de bacterias aisladas, adaptadas en este trabajo para su medida en muestras de sedimento.

Inicialmente, la muestra de sedimento superficial seco (A) se humidificó con agua Milli-Q hasta un contenido de humedad del 30%, y se incubó a 13°C (temperatura media del agua del río) durante dos días para la aclimatación de los microorganismos. A continuación, se siguió el protocolo definido por Antolín et al., (2010) para la extracción enzimática en plantas, pero con modificaciones establecidas en el Ensayo 1 (Apartado 4.2.1) para su adaptación al sedimento. Primeramente, se mezclaron en un recipiente de polipropileno 1 g de muestra de sedimento superficial y 12 mL de una solución tampón fosfato potásico 50 mM (pH = 7,5) + EDTA 2 mM + DTT 2 mM + PVPP insoluble (1%, m/v) + caseína soluble (1,5%, m/v) a 0°C. Posteriormente, el recipiente se tapó y se dispuso en un agitador rotatorio durante 10 minutos. Finalmente, se centrifugó a 4300 r.p.m durante 5 minutos y el sobrenadante (en adelante extracto crudo) se conservó en hielo hasta su análisis.

La determinación de la **actividad enzimática Nitrato Reductasa (NR)** se realizó de acuerdo con el protocolo definido por Antolín et al., (2010) para plantas, pero con modificaciones establecidas en el Ensayo 2 (Apartado 4.2.2) para su adaptación al sedimento. Inicialmente, se adicionaron en un tubo de ensayo 0,5 mL de extracto crudo, 0,1 mL de solución tampón fosfato potásico 50 mM (pH = 7,5), 0,1 mL de una disolución de NADH (1 mg L^{-1}), 0,2 mL de una disolución de KNO_3 100 mM y agua Milli-Q hasta un volumen final de 2 mL. Tras 1 h de incubación de la mezcla a 28°C en la oscuridad, la reacción se paró sumergiendo los tubos de ensayo en un baño de hielo. Se tomó una alícuota de 0,8 mL y se diluyó con agua Milli-Q hasta un volumen final de 10 mL. Para cuantificar el contenido de NO_2^-, se añadieron 1 mL de disolución de sulfanilamida (1%, m/v) en HCl 1,5 M y 1 mL de disolución de N-(1-naftil)-etilendiamina diclorhidrato (0,1%, m/v), midiendo tras 15 minutos la absorbancia (λ = 540 nm) mediante espectrofotometría (adaptación del método 354.1 de USEPA, 1979). La actividad enzimática Nitrato Reductasa se expresó como los µmoles de NO_2^- generados por g de sedimento seco y hora de incubación.

La determinación de la **actividad enzimática Nitrito Reductasa (NiR)**, se realizó de acuerdo con el protocolo definido por Kakutani et al., (1981) para cultivos de bacterias aisladas, pero con modificaciones establecidas en el Ensayo 2 (Apartado 4.2.2) para su adaptación al sedimento. Inicialmente, se adicionaron en un tubo de ensayo 0,1 mL de extracto crudo y 0,8 mL de una solución tampón fosfato potásico 25 mM (pH = 7) + KNO_2 2,5 mM + metil viológeno 125 µM. Tras 2 minutos de pre-incubación de la mezcla a 30°C, la reacción se inició con la adición de 0,1 mL de una disolución $NaHCO_3$ 0,1 M y $Na_2S_2O_4$ 5 mM. Tras 90 minutos de incubación de la mezcla a 30°C en la oscuridad, la reacción se paró agitando vigorosamente la mezcla en un vórtex hasta la desaparición de la coloración azul y sumergiendo los tubos de ensayo en un baño de hielo. Se tomó una alícuota de 0,6 mL y se diluyó con agua Milli-Q hasta un volumen final de 10 mL. Una vez cuantificado el contenido de NO_2^- (USEPA, 1979), la actividad enzimática Nitrito Reductasa se expresó como los µmoles de NO_2^- consumidos por g de sedimento seco y hora de incubación.

4.3.2.2. Bioindicadores de biodiversidad microbiana

Para analizar la biodiversidad (funcional y estructural) de las comunidades bacterianas presentes en los sedimentos superficiales se combinaron dos técnicas moleculares: la PCR cuantitativa (en inglés, quantitative polymerase chain reaction (qPCR)) y el metabarcoding del gen 16S ARNr (en inglés, rRNA). La realización de estos análisis tuvo lugar gracias a un convenio de colaboración entre la UPV-EHU y los Centros de Investigación Tecnológicos de la Red Vasca de Ciencia y Tecnología, en el cual está incluido NEIKER-TECNALIA (Departamento de Conservación de Recursos Naturales, Derio).

Para la determinación de la diversidad funcional de bacterias desnitrificantes, se comenzó extrayendo el ADN de 0,25 g (peso seco) de muestra de sedimento superficial almacenada fresca a -20°C (C), mediante el kit Power Soil™ DNA Isolation Kit. Previamente, las muestras se lavaron dos veces en K_2HPO_4 120 mM (pH 8,0) para retirar el ADN extracelular. La muestra se centrifugó a alta velocidad (13.000 g), de modo que el sobrenadante (ADN extracelular disuelto en solución de K_2HPO_4) se eliminó mientras que las células permanecieron aún intactas en la muestra de sedimento (Kowalchuk et al., 2003).

A continuación, se preparó una librería metabarcoding (amplicones) de acuerdo con

Lanzén et al. (2016). El gen de la subunidad menor del ARN ribosómico de procariotas (16S rRNA) fue amplificado mediante los primers 519F y 806R dirigidos a la región hipervariable V4 (Tabla 4.1). Durante la primera PCR se utilizó una pareja de primers con adaptadores, continuando con una limpieza y otra PCR de 27 ciclos con adaptadores unidos a códigos de barras específicos de muestra (en inglés, sample specific barcodes). Finalmente, los extremos fueron secuenciados mediante Illumina MiSeq con el kit V2 de Tecnalia Corporation (Miñano, España). Los datos de secuenciación se depositaron en el Archivo Europeo de Nucelótidos (European Nucleotide Archive) con número de acceso PRJEB24857.

Tabla 4.1. *Set de primers empleado en la amplificación del gen objetivo en la qPCR y metabarcoding*

Gen	Primer	Secuencia (5' - 3')	Referencias
Metabarcoding del gen 16S rRNA			
16S rRNA	519F	CAGCMGCCGCGGTAA	Adaptado de Øvreås et al., 1997
	806R	GGACTACHVGGGTWTCTAAT	D'Amore et al., 2016
qPCR			
16S rRNA	1055F 1392R	ATGGCTGTCGTCAGCT ACGGGGCGGTGTGTAC	Huang et al., 2011
nirK	F1aCu R3Cu	ATCATGGT(C/G)CTGCCGCG GCCTCGATCAG(A/G)TTGTGGTT	Deslippe et al., 2014
nirS	cd3aF R3cd	GT(C/G)AACGT(C/G)AAGGA(A/G)AC(C/G)GG GA(C/G)TTCGG(A/G)TG(C/G)GTCTTGA	Throbäck et al., 2004
nosZ	Nos661F Nos1773R	CGGCTGGGGGCTGACCAA ATRTCGATCARCTGBTCGTT	Braker et al., 2011

La abundancia de bacterias totales (gen 16S rRNA) y de genes desnitrificantes (*nirK, nirS y nosZ*) se cuantificó mediante qPCR de acuerdo con Epelde et al. (2014). Los valores de abundancia fueron corregidos con sus respectivos porcentajes de eficiencia de la PCR (16S rRNA, 98,82%; *nirK*, 96,82%; *nirS*, 92,17%;y *nosZ*, 95,02%). Además, el límite de detección se estableció en función de los valores más altos de ciclos umbrales (en inglés, cycle threshold (Ct)) cuantitativamente determinados (Ct: 16S rRNA, 29,65; *nirK*, 31,48; *nirS*, 33,24; y *nosZ*, 28,60), y la especificidad de las PCRs se revisó mediante las curvas de disociación (en inglés,

melt curve). Asimismo, los patrones empleados se elaboraron a partir del genoma extraído de cultivos bacterianos puros, como se describe en Yergeau et al. (2007): *Escherichia coli* para los genes 16S rRNA, *nirK* y *nirS*, y *Pseudomonas mendocina B* para el gen *nosZ*. Los primers utilizados se detallan en la Tabla 4.1. Cada 25 µl de mezcla de reacción contenían 2,5 µl de ADN, 12,5 µL de SYBR PremixExTaq (Takara), 2,5 µL de cada primer en una concentración de 10 µM, 1,25 µL de albúmina de suero bovino (BSA; 40 mg mL^{-1}) y 0,5 µL de colorante ROX. Las condiciones de la qPCR para el gen 16S rRNA fueron: 95ºC durante 15 min; 94ºC durante 30 s; 52ºC durante 30 s; 72ºC durante 1 min (40 ciclos); 95ºC durante 15 s; 60ºC durante 1 min; y 95ºC durante 30 s para la curva de disociación (melt curve), con una extensión final de 60ºC durante 15 s. Las condiciones de la qPCR para los genes *nirK*, *nirS* y *nosZ* fueron: 95ºC durante 15 s; 60ºC durante 1min; 95ºC durante 30 s para la curva de disociación (melt curve), con una extensión final de 60ºC durante 15 s.

Los pares de lectura de secuencia (en inglés, sequence read-pairs) se filtraron por calidad y se solaparon mediante la herramienta vsearch con los parámetros establecidos por defecto, excepto los pares de lectura "escalonados" (Rognes et al., 2016). Utilizando la herramienta informática Cutadapt (Martín, 2011), se recortaron los extremos de ambos pares de lectura para eliminar el N5 y las secuencias de los primers, descartando así cualquier secuencia que no contuviese el primer completo permitiendo un desemparejamiento. Finalmente, utilizando nuevamente vsearch, las secuencias se truncaron a 253 nt, eliminando aquellas más cortas o con baja calidad (fastq_maxee = 0,5). Todas las secuencias superpuestas con filtrado de calidad satisfactorio se agruparon en unidades taxonómicas operativas (en inglés Operational Taxonomic Units, OTUs) utilizando el programa Swarm v2 (Mahé et al., 2015). Las OTUs "swarm" se sometieron a un filtrado quimérico de referencia y de novo (con la base de datos de referencia rdp_gold), usando vsearch (algoritmo UCHIME). A continuación, se agruparon nuevamente en OTUs mediante vsearch, considerando las lecturas de abundancia totales y utilizando un umbral de divergencia de secuencia máximo del 3% (Rognes et al., 2016). Las abundancias de OTU se obtuvieron mediante el re-mapeo de las lecturas a las secuencias de OTU representativas.

La clasificación taxonómica se realizó alineando secuencias OTU representativas con la base de datos SilvaMod (v128) utilizando blastn (v.2.2.25 + task megablast) y LCAClassifier

de CREST con parámetros predeterminados (Lanzén et al., 2012). Las OTUs no clasificadas por debajo del umbral de alineación y aquellas clasificadas como pertenecientes a genes de ARNr de orgánulos eucarióticos, se excluyeron del análisis subsecuente. Los análisis estadísticos se basaron en las abundancias relativas de taxones derivadas de CREST. Para todos los análisis que comparon la estructura de la comunidad con parámetros fisicoquímicos, se agruparon *in silico* las dos réplicas técnicas de metabarcoding para cada punto.

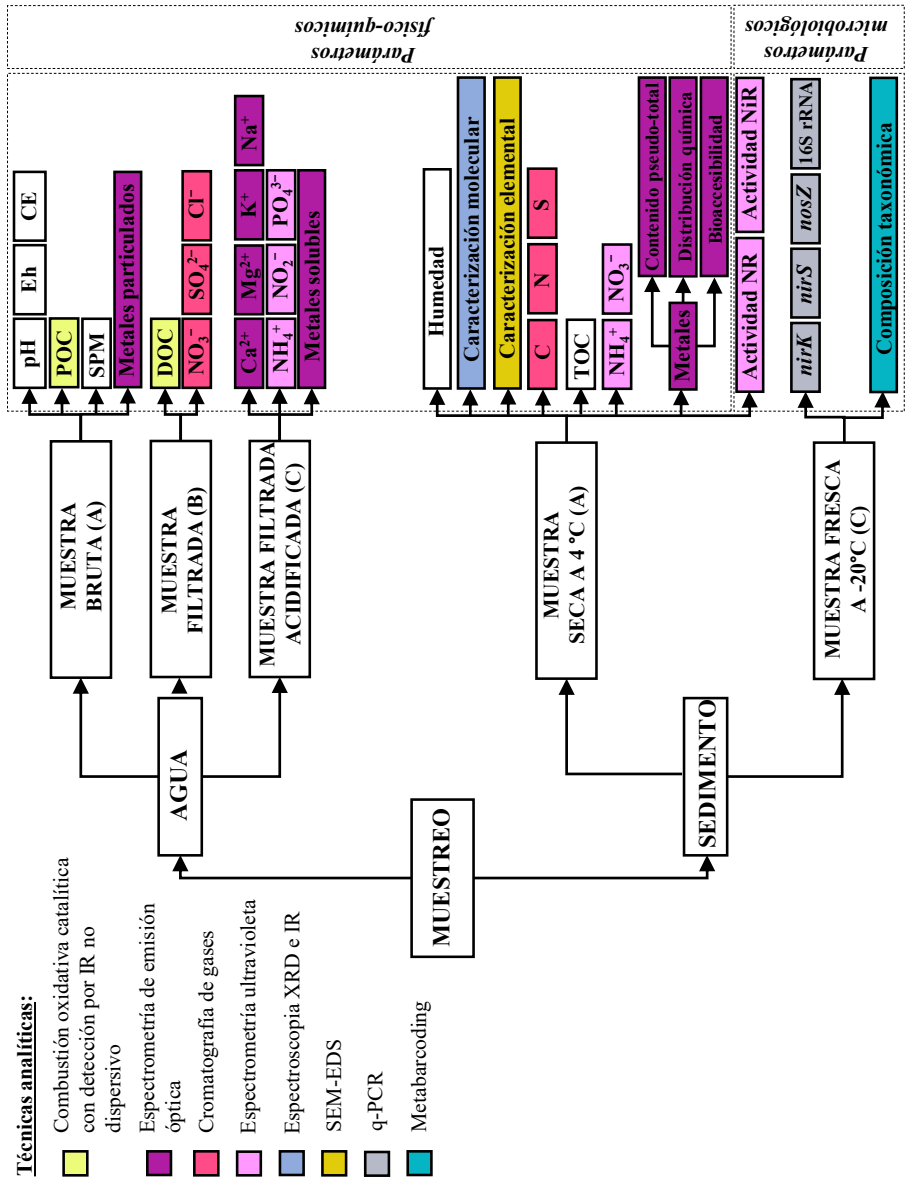

Fig. 4.3. *Esquema de todos los métodos analíticos (incluídas las técnicas analíticas) descritos previamente*

4.4. Evaluación de la calidad

En la Tabla 4.2, se recopilan todos y cada uno de los índices empleados a lo largo de este trabajo (Capítulos 5, 6, 7, 8 y 9) para evaluar la calidad de las muestras de sedimento superficial, a partir de los parámetros fisicoquímicos y biológicos.

Tabla 4.2. *Índices para la evaluación de la calidad de los sedimentos superficiales*

Índice	Función	Fórmula
Factor de divergencia (en inglés **Divergence Factor, DF**)	Identificar el grado de desviación del contenido pseudo-total de metales en el sedimento superficial de un punto de muestreo en el río ([M]) con respecto a la tendencia dominante (Mediana [M]) de toda la cuenca.	$DF = \sum_{i=1}^{i=7} \left(\frac{[M]}{\text{Mediana } [M]} \right)_i$ (4.1) i: número de metales considerados.
Factor de enriquecimiento (en inglés **Enrichment Factor, EF**); Factor de enriquecimiento global (en inglés **Global Enrichment Factor, GEF**)	Identificar el nivel de enriquecimiento de metales en el sedimento por contribución de fuentes antropogénicas frente a la contribución por fuentes naturales.	$EF = \frac{([M]_S/[R]_S)}{([M]_{rf}/[R]_{rf})}$ (4.2) $\quad GEF = \sum_{n=1}^{i=6} EF_i$ (4.3) $[M]_S$ y $[R]_S$: contenido pseudo-total del metal (i) y del elemento de referencia en el sedimento, respectivamente. $[M]_{rf}$ y $[R]_{rf}$: contenidos en la corteza continental superior.
Factor de contaminación individual (en inglés **Individual Contamination Factor, ICF**); Factor de contaminación global (en inglés **Global Contamination Factor, GCF**)	Identificar el riesgo de contaminación ambiental en relación con el tiempo de retención de los metales presentes en el sedimento superficial.	$ICF = \frac{C_{\text{no residual}}}{C_{\text{residual}}}$ (4.4) $\quad GCF = \sum_{n=1}^{i=7} ICF_i$ (4.5) C no residual: suma del contenido de metal (i) extraído en la primeras tres etapas (F_1, F_2 y F_3) de la extracción secuencial. C residual: contenido de metal extraído en la etapa F_4.
Cociente de peligrosidad (en inglés **Hazard Quotient, HQ**)	Estimar la capacidad de los metales para causar efectos adversos (no cancerígenos) sobre la población debido a una ingestión accidental de sedimento	$HQ = \frac{CDI \times B/100}{RfD}$ (4.6) CDI: dosis de ingestión diaria (mg g^{-1} $día^{-1}$). B: porcentaje de metal bioaccessible. RfD: dosis de referencia oral (mg g^{-1} $día^{-1}$)
Tiempo de respuesta (en inglés **Response Time, RT**)	Estimar la capacidad de los microorganismos presentes en el sedimento para desnitrificar el nitrato presente en el mismo.	$RT(\text{min}) = \frac{NO_3^- \text{en el sedimento } (\mu g\ N\ g^{-1})}{\text{Actividad NR } (\mu g\ N{-}NO_2^-\ \text{generado}\ g^{-1} \text{min}^{-1})}$ (4.7)
***nosZ*:nir**	Identificar una posible emisión de gas invernadero (N_2O) como producto de una desnitrificación incompleta en el sedimento	$\text{nosZ: nir} = \frac{\text{Abundancia gen } nosZ}{\text{Abundancia gen } nirS + \text{gen } nirK}$ (4.8)

Adicionalmente, como parte de uno de los objetivos de este trabajo, inferir un nuevo sistema de control del estado ecológico de la cuenca, se desarrolló un **índice de calidad** (en inglés **Quality Index, QI**) multimétrico tanto para el agua como para el sedimento:

$$QI = K - \sum_{j=1}^{n} w_j \, x \, M_{i,j} \tag{4.9}$$

donde K es una constante, w_j se corresponde con el peso atribuido a cada parámetro fisicoquímico (j) y $M_{i,j}$ es el valor (transformado logarítmicamente) del parámetro monitoreado en cada punto de muestreo (i). Si bien el QI es de carácter fisicoquímico, su desarrollo se sustentó en la infomación aportada por los índices de naturaleza biológica Tiempo de Respuesta (Response Time, RT) y ratio *nosZ*:nir, descritos en la Tabla 4.2.

Conjuntamente, y a partir de la expresión del QI, se propuso el cálculo del **Porcentaje de Contribución** (en inglés **Contribution Percentage, CP**) de cada parámetro fisicoquímico al índice de calidad, con el propósito de reconocer la susceptibilidad de una localización a lo largo del río debido a un valor anómalo de este parámetro en el agua y/o el sedimento:

$$CP_j\,(\%) = \frac{w_j x \dfrac{M_{i,j} - \bar{M}_j}{\sigma_j}}{\sum_{j=1}^{n} ABS\left| w_j x \dfrac{M_{i,j} - \bar{M}_j}{\sigma_j} \right|} x\, 100 \tag{4.10}$$

donde w_j cuantifica la influencia (magnitud y signo) del parámetro fisicoquímico (j) sobre la calidad del agua y/o sedimento, mientras que el valor monitoreado ($M_{i,j}$), normalizado con su media y desviación estándar (σ_j) a lo largo de toda la cuenca, permite identificar el grado de desviación de la concentración del parámetro en el punto de muestreo (i) considerado de la tendencia dominante de la cuenca.

El desarrollo estadístico para la obtención de estos índices, cuya base se centra en vincular los parámetros fisicoquímicos con parámetros microbiológicos, se encuentra pormenorizadamente descrito en el Capítulo 9.

4.5. Métodos estadísticos

A continuación, se detallan todas las técnicas estadísticas empleadas a lo largo de este trabajo (Capítulos 5, 6, 7, 8 y 9) para el procesamiento de datos.

Las técnicas descritas seguidamente se llevaron a cabo utilizando el paquete estadístico SPSS 22.0 (IBM):

a) **Test de Shapiro-Wilk** para examinar la distribución de probabilidad de las variables contínuas.

b) **Test de Levene** para evaluar la igualdad de varianza de las variables consideradas entre dos o más casos.

c) **Análisis Cluster o análisis de conglomerado jerárquico** para la clasificación de casos en función de la proximidad de las variables consideradas para cada uno de ellos. Como medida de proximidad se utilizó la distancia euclídea entre las variables, mientras que los casos fueron agrupados mediante el método de Ward.

d) **Análisis de Componentes Principales** (en inglés, Principal Component Analysis (**PCA**)) para la exploración de casos en función de múltiples variables seleccionadas para cada uno de ellos. Los componentes principales obtenidos se sometieron a rotación ortogonal varimax para maximizar la varianza y lograr que cada uno de ellos presentara altas correlaciones con unas pocas variables y correlaciones nulas con el resto. Se consideraron únicamente aquellos componentes principales (vectores propios) con un valor propio igual o mayor que 1.

e) **Correlaciones de Spearman** (test no paramétrico) para evaluar la interdependencia entre dos variables. Se consideró que la correlación era significativa cuando el coeficiente de correlación (en adelante ρ) fue mayor que 0,5 (para una interdependencia positiva) o menor que -0,5 (para una interdependencia negativa).

f) **Análisis de la Varianza** (en inglés, Analysis of Variance (**ANOVA**)) para determinar si las medias (intervalo de confianza del 95%) de la variable seleccionada eran

significativamente diferentes entre dos o más casos. Una vez determinada la existencia de diferencias significativas, la aplicación de **la prueba post-hoc de Tukey** permitió identificar qué casos diferían significativamente entre sí ($\rho < 0,05$). Para aplicar esta técnica estadística, la variable tenía que seguir una distribución normal en cada caso y todos ellos debían presentar igual varianza de la variable.

g) **La prueba de U-Mann Whitney** (prueba no paramétrica) para determinar si las medias de la variable seleccionada eran significativamente diferentes ($\rho < 0,05$) entre dos casos independientes.

h) **Análisis de regresión lineal** tanto simple como múltiple para explorar y cuantificar la dependencia de una variable con respecto a otra/s variables predictora/s. La bondad del ajuste lineal se determinó de acuerdo con el coeficiente de determinación R^2, que determina el porcentaje de variable dependiente que queda explicada por la/s variable/s predictora/s. Para contrastar si el modelo lineal obtenido era significativo ($\rho < 0,05$) se realizó conjuntamente un **análisis de la varianza de Fisher**.

Seguidamente se describen las técnicas estadísticas multivariantes de ordenación empleadas para el estudio de las comunidades biológicas en el sedimento (Capítulo 9), en el entorno del lenguaje R (paquete estadístico Vegan):

a) **Estimación de los índices de biodiversidad procariota (índice de Shannon (S') e índice de equidad de Pielou (H')) y riqueza rarefaccionada** a partir de las OTUs obtenidas en el metabarcoding del gen 16S rRNA (Oksanen et al., 2013).

b) **Análisis de la Varianza** (en inglés, Analysis of Variance (**ANOVA**)) junto con **la prueba post-hoc de Tukey** para detectar diferencias significativas en la diversidad alfa y abundancia relativa de OTUs entre dos o más casos, o agrupaciones de éstos según distintos criterios establecidos.

c) **Correlaciones de rango de Kendall** para estimar el nivel de asociación no paramétrica entre variables explicativas continuas que no seguían una distribución normal.

d) **Prueba de Bonferroni** para contrarrestar el error en las comparaciones múltiples y corregir la significancia (ρ) obtenida en las diferentes pruebas de contraste de hipótesis.

e) **Escalado multidimensional no métrico** (en inglés, non-metric multidimensional scaling (**NMDS**)) para la exploración conjunta de casos en función de parámetros fisicoquímicos y microbiológicos. Inicialmente, se ejecutó la función *metaMDS*, que trabaja con la matriz de disimilitudes (**Bray-Curtis**) de la composición microbiana procariota (gen 16S rRNA) obtenida a partir de las abundancias relativas de OTUs (previa transformación de **Hellinger**). A continuación, los vectores de las variables fisicoquímicas y microbiológicas se insertaron en el espacio geométrico NMDS mediante la función *envfit*. La función *bioenv* se utilizó para seleccionar el mejor subconjunto de variables, de modo que la matriz de distancias euclídeas de estas variables una vez normalizadas (promedio 0 y desviación estándar 1) tuvieran la máxima correlación con la matriz de disimilitudes (**Bray-Curtis**) de la composición microbiana. Por último, la función *adonis* permitió evaluar la significancia (ρ) de las correlaciones entre las matrices de disimilitudes/distancias aplicando el test permutacional de Mantel y el test parcial de Mantel.

f) **Análisis permutacional de la varianza** (en inglés, Permutational Analysis of Variance (**PERMANOVA**)) basado en matrices de distancia para detectar diferencias significativas (ρ) en la respuesta simultánea de las variables fisicoquímicas y microbiológicas a uno o varios factores.

4.6. Referencias

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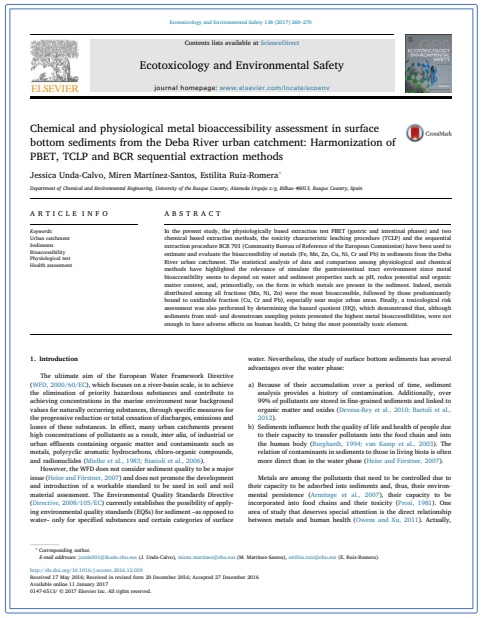
Results and discussion I

Chemical and physiological metal bioaccessibility assessment in surface bottom sediments from the Deba River urban catchment: harmonization of PBET, TCLP and BCR sequential extraction methods



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ABSTRACT

In the present study, the physiologically based extraction test PBET (gastric and intestinal phases) and two chemical based extraction methods, the toxicity characteristic leaching procedure (TCLP) and the sequential extraction procedure BCR 701 (Community Bureau of Reference of the European Commission) have been used to estimate and evaluate the bioaccessibility of metals (Fe, Mn, Zn, Cu, Ni, Cr and Pb) in sediments from the Deba River urban catchment. The statistical analysis of data and comparison among physiological and chemical methods have highlighted the relevance of simulate the gastrointestinal tract environment since metal bioaccessibility seems to depend on water and sediment properties such as pH, redox potential and organic matter content, and, primordially, on the form in which metals are present in the sediment. Indeed, metals distributed among all fractions (Mn, Ni, Zn) were the most bioaccessible, followed by those predominantly bound to oxidizable fraction (Cu, Cr and Pb), especially near major urban areas. Finally, a toxicological risk assessment was also performed by determining the hazard quotient (HQ), which demonstrated that, although sediments from mid- and downstream sampling points presented the highest metal bioaccessibilities, were not enough to have adverse effects on human health, Cr being the most potentially toxic element.

1. Introduction

The ultimate aim of the European Water Framework Directive (WFD, 2000/60/EC), which focuses on a river-basin scale, is to achieve the elimination of priority hazardous substances and contribute to achieving concentrations in the marine environment near background values for naturally occurring substances, through specific measures for the progressive reduction or total cessation of discharges, emissions and losses of these substances. In effect, many urban catchments present high concentrations of pollutants as a result, *inter alia*, of industrial or urban effluents containing organic matter and contaminants such as metals, polycyclic aromatic hydrocarbons, chloro-organic compounds, and radionuclides (Mielke et al., 1983; Brunoli et al., 2006).

However, the WFD does not consider sediment quality to be a major issue (Heise and Förstner, 2007) and does not promote the development and introduction of a workable standard to be used in soil and soil material assessment. The Environmental Quality Standards Directive (Directive, 2008/105/EC) currently establishes the possibility of applying environmental quality standards (EQSs) for sediment –as opposed to water– only for specified substances and certain categories of surface water. Nevertheless, the study of surface bottom sediments has several advantages over the water phase:

a) Because of their accumulation over a period of time, sediment analysis provides a history of contamination. Additionally, over 99% of pollutants are stored in fine-grained sediments and linked to organic matter and oxides (Devesa-Rey et al., 2010; Bartoli et al., 2012).
b) Sediments influence both the quality of life and health of people due to their capacity to transfer pollutants into the food chain and into the human body (Burghardt, 1994; van Kamp et al., 2003). The relation of contaminants in sediments to those in living biota is often more direct than in the water phase (Heise and Förstner, 2007).

Metals are among the pollutants that need to be controlled due to their capacity to be adsorbed into sediments and, thus, their environmental persistence (Armitage et al., 2007), their capacity to be incorporated into food chains and their toxicity (Prusi, 1981). One area of study that deserves special attention is the direct relationship between metals and human health (Owens and Xu, 2011). Actually,

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Metals are among the pollutants that need to be controlled due to their capacity to be adsorbed into sediments and, thus, their environmental persistence (Armitage et al., 2007), their capacity to be incorporated into food chains and their toxicity (Prosi, 1981). One area of study that deserves special attention is the direct relationship between metals and human health (Owens and Xu, 2011). Actually, metals in sediments can be transferred to humans via ingestion (Jamshidi-Zanjani et al., 2014), dermal contact (Swarnalatha et al., 2015) or breathing (Yager et al., 2015), but only the fraction that is biologically available is able to cause harmful effects. However, the EQSs established in Directive 2008/105/EC are expressed as a total concentration and lead to an overestimation of the health hazard, since metals in sediments exist in different chemical forms or types of binding, affecting their mobility, bioavailability and toxicity (Yuan et al., 2004; Kordel et al., 2013). Thus, a special consideration of sediments and investigations on bioavailability of metals will be critical to properly identifying the level of pollution in rivers and aquatic environments.

Bioavailability may be defined as the degree to which chemicals present in the sediment are available for absorption into the systemic circulation system. It is therefore a comprehensive process that includes: (i) availability of the contaminant in the sediment (environmental availability) and release of the contaminant from sediment into the organism (bioaccessibility), (ii) uptake of the contaminant by the organism, and (iii) accumulation and/or effect of the contaminant within the organism (Lanno et al., 2004; Harmsen, 2007). In practice, bioavailability may be evaluated by performing in vivo experiments with animals that are anatomically, metabolically, and physiologically similar to humans (Drexler and Brattin, 2007; Turner, 2011). An ethical alternative for evaluating bioavailability is to assess bioaccessibility by in vitro methods, which provides the amount of chemical that is available for

solubilisation but not necessarily available for subsequent absorption through a physiological membrane. In this study, an in vitro physiologically based extraction test (PBET) has been used to provide metal bioaccessibility from sediments due to human oral uptake, simulating physiological conditions depending on the gastrointestinal segment studied (residence time, pH, composition of digestive solutions, solid-to-liquid ratio, temperature, mixing type and rate, aerobic/anaerobic conditions and food addition). To date, a strong correlation has been seen between results obtained using the PBET method and in vivo studies involving Pb or As-contaminated soils (Juhasz et al., 2013; Li et al., 2015). However, despite studies focuses on bioaccessibility assessment for other typical urban pollutants on soils such as Ni, Cr, Cu or Zn have proliferated (Madrid et al., 2007a; Madrid et al., 2007b; Poggio et al., 2009), limited in vivo data are currently available in the literature validating PBET as bioavailability assessment method for metals on sediments. Therefore, in the absence of in vivo experiments on sediments for bioaccessibility data validation, in this study chemical extraction methods have also been proposed to link bioaccessibility to leachability and metal fractionation in accordance with the recommendations of International Standard ISO 17402:2008 and other investigations done in soils and sediments (Devesa-Rey et al., 2008; Rodriges et al., 2014; Ren et al., 2015), in which the easily extractable metals are equate with those that are likely to be bioaccessible. Moreover, wider application of various accepted in vitro methods to sediment samples would provide greater understanding of the factors governing metal distribution and bioaccessibility.

In this context, the main aim of this study is to use a chemical method and a physiological method to evaluate metal bioaccessibility in surface bottom sediments from the Deba River catchment. The partial objectives are i) to demonstrate that metal bioaccessibility assessment should be physiologically based mimicking the human gastrointestinal environment, by comparing the TCLP chemical method and the PBET physiological procedure, ii) to examine whether PBET is applicable for assessing bioavailability of the metals selected, relating metal fractionation with metal bioaccessibility results, iii) to evaluate the spatial and temporal evolution of metal bioaccessibilities and iv) to make a health risk assessment of sediments.

5.3. Material and methods

5.3.1. Study area

The Deba River catchment (538 km^2) is located on the north-east coast of Spain (Gipuzkoa, Basque Country, Fig. 1). The Deba River (60 km) runs across the catchment towards the Bay of Biscay, receiving inflows from several streams, including the Ego and Oñati, the two most important tributaries. The catchment, which has a maximum gradient of 40%, rises from 0 m at sea level to an elevation of 1320 m a.s.l. at the highest peak (Botreaitz). In this study, the Altzola gauging station (Fig. 1) was considered as the outlet of the catchment since the lower part of this catchment is influenced by seawater when the tide is high.

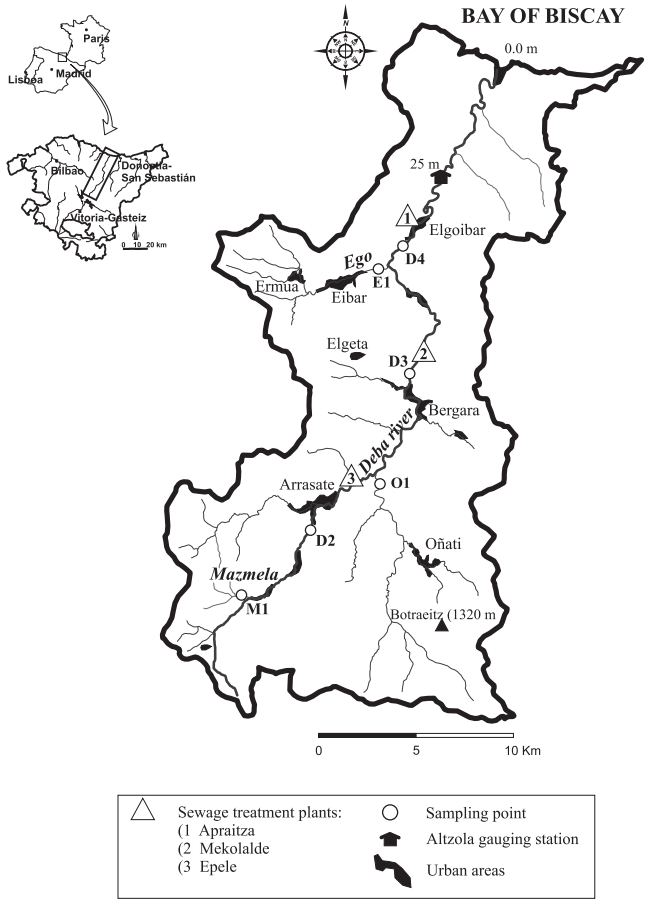

Fig. 5.1. *Location of the sampling points, Altzola gauging station and wastewater treatment plants in Deba River catchment*

Due to intense industrial development and a population increase along the river and its tributaries over recent decades, the Deba is considered to be one of the catchments most polluted by municipal and industrial wastewaters in the province of Gipuzkoa (Borja et al., 2006). The three most important urban areas are Eibar, Arrasate and Ermua, which contain 50% of all inhabitants. The primary industries in this catchment are metallurgy, automotive plants, galvanising, smelting and electrical appliance manufacture, which are concentrated in the urban areas along the river. In order to prevent river water pollution, three sewage treatment plants have been built in recent years to treat urban and industrial effluents before they are discharged into the river (Fig. 1). However, about 6773 m^3 y^{-1} of untreated industrial effluents are still discharged into the Deba River and its tributaries, and the Ego stream receives untreated wastewater inputs from Ermua. In addition, before the Epele plant came into continuous operation (May 2012), the organic-rich wastewaters of the towns of Arrasate and Oñati were discharged into the Deba River and Oñati stream via two sewers.

5.3.2. Field methodology

Surface bottom sediment (SBS) samples were collected from three sampling points (D2, D3 and D4) along the main river bank and from three sampling points (M1, O1 and E1) in the Mazmela, Oñati and Ego tributaries, respectively, during the sampling campaigns of October 2011 and October 2012. These sampling points were chosen in order to study the influence of anthropogenic pollution sources in the main river and tributaries on metal bioaccessibility from headwaters to the outlet. According to USEPA 2001a, SBS subsamples from multiple points within each sampling site were collected using a plastic spoon, composited in the field and sealed in clean polypropylene bags. All sediment samples were stored and refrigerated in the dark and transported to the laboratory of Chemical and Environmental Engineering (University of the Basque Country) on the same day. The pH and redox potential (Eh) of river water were measured *in situ* using a Crison Micro pH 2000 and Hach ORP/Redox sonde MTC101 with an Ag/AgCl electrode, respectively.

5.3.3. Laboratory methodology

SBSs were air-dried and ground with a pestle and mortar for homogenization. The

fine fraction of the sediment samples (< 63 μm) was sieved through a stainless steel sieve. This fraction has been proven to be the most chemically active sediment phase (Förstner and Salomons, 1980; Zhang et al, 2002).

To document the temporal and spatial patterns in metal contamination (Fe, Mn, Zn, Cu, Ni, Cr and Pb) in sediments, some authors (Martínez-Santos et al., 2015) have determined: (a) the pseudo total metal content using an ETHOS 1, Millestone microwave digestion system, where three replicates of each SBS sample (0.5 g) were heated in Teflon vessels with concentrated HNO_3:$HClO_4$ (3:1.5) by increasing the temperature to 180 ºC for 10 min and kept at that temperature for an additional 25 min (USEPA, 2007) and, (b) the metal distribution by a sequential extraction of metals using the European Community Bureau of References (BCR 701) procedure, which is divided into four operationally defined fractions: (i) exchangeable and acid-extractable (F_1, soluble species, carbonates and exchangeable metals), (ii) reducible (F_2, Fe/Mn oxyhydroxides), (iii) oxidizable (F_3, organic matter and sulphides), and (iv) residual fraction (F_4, remaining, non-silicate bound metals). In addition, total carbon (TC), nitrogen (TN), and sulphur (TS) were analysed in the fine sediment using a TruSpec CHNS determinator (Leco Corporation). Volatile solids were determined using a muffle furnace as described in Method 2540 E of the Standard Methods (APHA, 2015). After incineration at 500 °C, the percentage of weight loss was considered to be representative of total organic carbon (TOC). For controlling analytical methods a NBS sediment sample was additionally used (Buffalo River sediment, USA). With this technique, all metals were also measured with mean values close to the certified contents and variation coefficients lower than 8% with the exception of Pb (17% with 0.5 g of sample).

Although it is often used to evaluate the leachability of solid waste in the natural environment, in this study a toxicity characteristic leaching procedure (TCLP) is applied to evaluate metal bioaccessibility in sediments in accordance with Method 1311 (USEPA, 1993). Three replicates of each SBS sample (1g) of the fine sediments were continuously shaken at 30 r.p.m for 18 h at 20-22ºC with 20 mL (0.1 M) of acetic acid. After extraction, the extractable fraction was separated by centrifugation (7000240 Selecta) at 960 g for 20 min. The supernatant was filtered through a 0.45 μm Millipore nitrocellulose filter and stored in polyethylene bottles in the refrigerator (4 °C) as above, following addition of 40 μL of

concentrated HNO_3 to adjust the pH to below 2. The metal bioaccessibility (B) was calculated as shown in Eq. (5.1):

$$B(\%) = \frac{\text{Metal in supernatant, mg } L^{-1}}{\text{Metal in SBS, mg } g^{-1} \text{ x } \frac{1g}{0.02L}} \text{ x } 100 \quad (5.1)$$

A physiologically based extraction test (PBET) is an *in vitro* procedure developed to determine metal concentration in soils that could be absorbed through digestion in the human upper gastrointestinal tract (Ruby et al., 1993). However, this test could be adapted to other matrices like sediments (Devesa-Rey et al., 2010), dust (Bi et al., 2015) or food (Llorente-Mirandes et al., 2016).The test is designed around realistic parameters (solid-to-liquid ratio, mixing rate, pH and chemistry) of a 2-to-3 year old child, considered to be the subject at greatest risk to metal exposure from accidental ingestion. In the current study, the extraction method is based on the procedure designed by Ruby et al. (1993) but modified by Drexler, who included improvements applied also in the standard methodology in accordance with the Environmental Protection Agency (USEPA, 2007).

The gastric solution of the PBET was prepared by adding 0.625 g of pepsin, 0.25 g of sodium citrate, 0.25 g of sodium malate, 210 µL of lactic acid and 250 µL of acetic acid to a 500 mL flask, and diluting the content with Milli-Q water. The pH of the gastric solution was subsequently adjusted to 2.5 by drop wise addition of concentrated HCl. Three replicates of each SBS sample (0.5 g of the fine fraction) were continuously shaken in an orbital shaker at 60 r.p.m for 1 h at 37ºC with 50 mL of gastric solution. After extraction, 20 mL of aliquot was pipetted and the extractable fraction was separated by centrifugation at 2000 r.p.m for 20 min. The supernatant was filtered through a 0.45 µm Millipore nitrocellulose filter ("End of stomach phase"). The slurries from the stomach phase were then continuously shaken at 60 r.p.m for 3 h at 37ºC with an additional 20 mL of gastric solution, 70 mg of bile salts, 20 mg of pancreatin and few mL of (1M) sodium bicarbonate to adjust to pH 7. After extraction, 20 mL of aliquot was pipetted and the extractable fraction was separated by centrifugation at 2000 r.p.m for 20 min. The supernatant was filtered through a Number 2 Whatman paper and then through a 0.45 µm Millipore nitrocellulose filter ("End of intestinal phase"). Until analysis, both supernatants were stored in polyethylene bottles in the refrigerator (4°C) as above after

the addition of concentrated HNO_3 to adjust the pH to below 2. The metal bioaccessibility (B) was calculated as shown in Eq. (5.2):

$$B(\%) = \frac{\text{Metal in supernatant, mg L}^{-1}}{\text{Metal in SBS, mg g}^{-1} \text{ x } \frac{0.05\text{g}}{0.05\text{L}}} \text{ x } 100 \quad (5.2)$$

Finally, the metals under consideration (Fe, Mn, Zn, Cu, Ni, Cr and Pb) in the supernatants were determined by ICP-OES (Perkin Elmer Optima 2000). The detection limit for these metals was: Pb (1 µg g^{-1}), Zn (0.5 µg g^{-1}), Fe and Mn (0.4 µg g^{-1}) and Cr, Cu and Ni (0.1 µg g^{-1}).

5.3.4. *Bioaccessibility assessment evaluation*

The bioaccessibility assessment evaluation ratio (BAER) was proposed in this study to evaluate whether the physiological based method is a better assessor of metal bioaccessibility on sediments than the chemical based procedures in the absence of bioavailability data for validation. The BAER was calculated as shown in Eq. (5.3):

$$\text{BAER} = \frac{\text{Extracted metal in PBET, µg g}^{-1}}{\text{Extracted metal in TCLP, µg g}^{-1}} \quad (5.3)$$

In order to check the relevance of gastrointestinal environment reproduction to metal bioaccessibility assessment, comparison of the data obtained with chemical extraction tests need to be carried out. This ratio is based on two ideas:

1) The consideration that the easily extractable metals for the chemical procedures are likely to be bioaccessible. In the case of TCLP, leachability is supposed to be equal to bioaccessibility. For the BCR, soluble species, carbonates and exchangeable metals (F_1) are assumed to be the bioaccessible fraction (Wragg and Cave et al., 2003; Devesa-Rey et al., 2008; Sundaray et al., 2011).

2) TCLP follows the same procedure as the first step (F_1) in BCR sequential extraction. Therefore, comparison between PBET and TCLP is only necessary.

The ranges of BAER values and assigned descriptions are shown in Table 5.1. Simulation of human gastrointestinal tract is therefore considered to be decisive for bioaccessibility assessment when the BAER value exceeds 1 (PBET extracts at least the metal fraction conceived as the most easily extractable only with pH variations).

Table 5.1. *BAER and HQ indexes*

Bioaccessibility Assessment Evaluation Ratio		
Log_{10}BAER	**Description**	
Log_{10} BAER < 0	Bioaccessibility < Leachability	Relevance of physiologically based gastrointestinal tract simulation unproven.
Log_{10} BAER = 0	Bioaccessibility = Leachability	
0 < Log_{10} BAER < 1	Bioaccessibility > Leachability	Relevance of physiologically based gastrointestinal tract simulation proven.
1 < Log_{10} BAER < 2	Bioaccessibility > 10 Leachability	
2 < Log_{10} BAER	Bioaccessibility > 100 Leachability	

Toxicity Risk Assessment				
Parameter	**Description**		**Value**	**Reference**
C (mg g^{-1})	Metal Pseudo-Total Concentration		In Reference	Martínez-Santos et al., 2015
I_R (mg day^{-1})	Sediment ingestion rate		200 (children)	USEPA, 2001b
E_F (day $year^{-1}$)	Exposure frequency		350	USEPA, 2001b
E_D (year)	Exposure duration		6 (children)	USEPA, 2001b
W_{AB} (kg)	Body weight		15 (children)	USEPA, 1989
T_A (day)	Averaging time		E_D·365	USEPA, 2001b
B (%)	Bioaccessibility		PBET Results	Table 5.2
RfD_o (mg (kg·day)$^{-1}$)	Oral Reference Dose	Fe	7.0 exp-01	USEPA, 2010
		Mn	1.4 exp-01	USEPA, 2010
		Zn	3.0 exp-01	USEPA, 2010
		Cu	4.0 exp-02	USEPA, 2010
		Ni	2.0 exp-02	USEPA, 2010
		Cr	3.0 exp-03	USEPA, 2010
		Pb	3.5 exp-03	Hu et al. 2011

5.3.5. Health risk assessment

The purpose of a hazard assessment for non-carcinogenic effects is to estimate the potential for metals to cause harmful effects in exposed people, especially children, due to accidental contaminated sediment ingestion (Calabrese et al., 1991; Maddaloni et al., 1998; Grøn and Andersen, 2003). For this purpose, the chemical daily intake (CDI) and hazard quotient (HQ) derived from US EPA were calculated as shown in Equations (5.4) and (5.5), respectively:

$$\mathrm{CDI}\left(\frac{\mathrm{mg}}{\mathrm{kg}\cdot\mathrm{day}}\right) = \mathrm{C}\ \mathrm{x}\ \frac{\mathrm{I_R}\ \mathrm{x}\ \mathrm{E_F}\ \mathrm{x}\ \mathrm{E_D}}{\mathrm{W_{AB}}\ \mathrm{x}\ \mathrm{T_A}} \tag{5.4}$$

$$\mathrm{HQ} = \frac{\mathrm{CDI}\ \mathrm{x}\ \mathrm{B}/100}{\mathrm{RfD}} \tag{5.5}$$

The parameters of those equations are shown in Table 5.1. While CDI determines the possible entry of metals into the human body, HQ compares released metal with the corresponding oral reference dose. RfD is the estimated amount of the daily oral exposure level for the population that is likely not to have an appreciable risk of deleterious effects during a lifetime. If the exposure level of a metal exceeds the corresponding RfD, *i.e.*, HQ exceeds 1, the SBS may be a concern for potential harmful effects.

5.3.6. Statistical analysis

A cluster analysis using squared Euclidean distance and aggregation Ward's method was performed to discriminate metal bioaccessibility measured using both the TCLP and PBET methods and to identify groups of metals with the same behaviour. In addition, principal component analysis (PCA) was performed to establish relationships between metal bioaccessibilities and other elements in the water and sediments. PCA with an eigenvalue greater than 1 was subjected to an orthogonal varimax rotation. This maximises the variance to obtain a pattern of loadings for each factor that is as diverse as possible, thus lending itself to easier interpretation. In addition, a Spearman correlation analysis (non parametric test) was performed to establish relationships between BCR sequential extraction and TCLP or PBET

methods, and to improve our understanding of the methodology used in this study. Finally, an analysis of variance (ANOVA) with analysis method type as a factor was performed to compare differences between TCLP and PBET at each sampling point using one-way ANOVA and Tukey's post-hoc test. The aim of this analysis is to demonstrate that an *in vitro* test is more applicable for bioaccessibility determination than the chemical method. Statistical processing of the data was performed using SPSS Software 22.0.

5.4. Results and discussion

5.4.1. Influence of nature of methodology on bioaccessibility assessment

Table 5.2 shows the mean Fe, Mn, Zn, Cu, Ni, Cr and Pb pseudo-concentrations, metal distribution and percentages (in parentheses) of the bioaccessibilities determined by TCLP and PBET (gastric and intestinal phases) methods in the fine sediments for all sampling sites and the two sampling periods. Martínez-Santos et al. (2015) found a clear trend of downstream increase in the concentration of trace metals (Zn, Cr, Cu and Ni) in sediments, evidencing the relatively unpolluted nature of the headwaters and contamination of the river in the mid- and low-water courses of this catchment due to untreated industrial effluents from galvanising, steel and metallurgical plants, among others, and untreated or partially-treated wastewater effluents. Moreover, Cu, Cr and Pb were found in the most potentially mobile fractions, predominantly bound to organic matter and/or sulphides (38.4-71.1%), and Mn, Ni and Zn distributed among all four fractions. In contrast, the largest proportion of Fe was associated with the residual fraction (>74.8%).

A Cluster Analysis was also carried out to establish different groups of metals according to their bioaccessibility and to identify different metal behaviour depending on those methods and phases for each sampling year. The results of the cluster analysis show the same pattern of metal groups for both years (Fig. 5.2), and two main groups can thus be distinguished:

a) One group includes Ni, Mn and Zn, which were the elements with the highest extractability in both methods and phases (18.7-44.5%, Table 5.2), except for Zn, whose bioaccessibility in the intestinal phase of PBET was one of the lowest (4.0-5.7%, Table 5.2). While Mn and Zn were more extracted in TCLP than in PBET (25.7-44.5%, Table

5.2), indicating that the acid environment presents the problem of re-adsorption of these dissolved metals at low pH (Turner and Olsen, 2000; Devesa-Rey et. al., 2010), Ni presented a higher bioaccessibility in the gastric phase of PBET (37.7-42.7%, Table 5.2).

b) The second group consists of the elements (Pb, Cu, Cr and Fe) with the lowest bioaccessibilities in all methods (0.60-15.4%, Table 5.2), except in the intestinal phase of PBET, where Zn was included as abovementioned. All these metals were extracted more in PBET than in TCLP (15.4-3.6%, Table 5.2) and could be separated into two sub-groups:

i) The first sub-group is exclusively composed of Pb, which showed the highest bioaccessibilities after Ni, Mn and Zn, except in the intestinal phase of PBET in 2011 (5.7%, Table 5.2).

ii) In the second sub-group (Fe, Cr and Cu), Cu was the only with higher bioaccessibility in the intestinal phase than in the gastric phase (7.6-9.3%, Table 5.2).

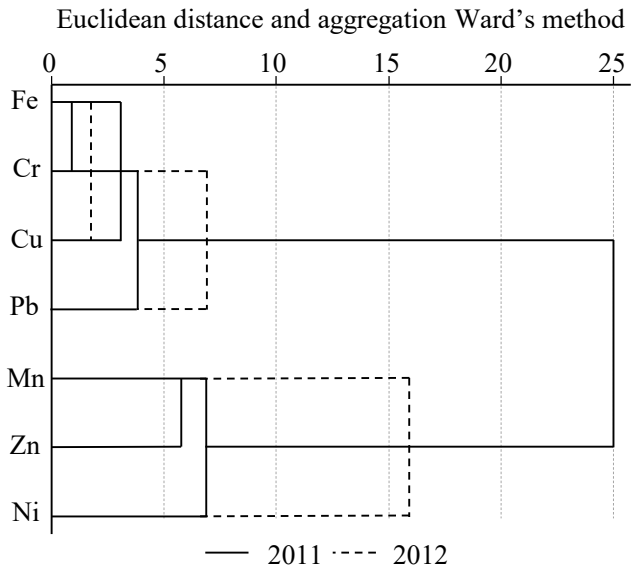

Fig. 5.2. *Cluster analysis of metals according to their bioaccessibility measured by PBET (gastric and intestinal phases) and TCLP for each sampling year*

Table 5.2. *Pseudo-total metal content, metal distribution determined by BCR and metal bioaccessibility determined by TCLP and PBET (gastric and intestinal phases) methods in fine sediments for all sampling sites (N = 6) of each year. The mean and standard deviation (± SD) data are presented in μg g^{-1} dry weight. The values in parentheses show percentage of elemental concentration, where the highest bioaccessibility percentage among methods (TCLP-PBET)/phases (gastric-intestinal) for each metal is shown in bold. The total content and metal distribution (μg g^{-1}) were determined by Martínez-Santos et al., (2015)*

Year	Metal	Total	BCR				TCLP	PBET	
			F_1	F_2	F_3	F_4		Gastric Phase	Intestinal Phase
2011	Fe	28790 ± 4539	885 ± 934 (3.3)	2968 ± 2368 (9.8)	2668 ± 381 (9.2)	23162 ± 4091 (78.2)	885 ± 934 (3.3)	1500 ± 822 (**5.4**)	1251 ± 668 (4.5)
	Mn	678 ± 252	339 ± 278 (44.5)	181 ± 239 (19.7)	53.7 ± 38.5 (9.9)	125 ± 43.0 (23.6)	339 ± 278 (**44.5**)	255 ± 127 (37.1)	142 ± 107 (18.7)
	Zn	2277 ± 1882	903 ± 827 (33.5)	983 ± 1095 (29.8)	576 ± 692 (26.1)	108 ± 68.2 (13.4)	903 ± 827 (**33.5**)	517 ± 372 (22.1)	867 ± 58.9 (4.0)
	Cu	197 ± 118	0.51 ± 0.63 (1.1)	6.5 ± 12.9 (5.1)	146 ± 115 (68.1)	28.7 ± 13.3 (25.6)	0.51 ± 0.63 (1.1)	4.3 ± 5.6 (6.2)	8.8 ± 5.9 (**7.6**)
	Ni	116 ± 79	52.6 ± 54.6 (33.5)	17.5 ± 16.8 (12.4)	15.7 ± 6.5 (18.1)	26.9 ± 8.1 (35.1)	52.6 ± 54.6 (33.5)	55.6 ± 51.5 (**37.7**)	34.1 ± 30.4 (24.3)
	Cr	309 ± 251	6.8 ± 7.5 (1.6)	33.0 ± 12.9 (5.1)	146 ± 115 (68.1)	28.7 ± 13.3 (25.6)	6.8 ± 7.5 (1.6)	41.9 ± 32.8 (**11.2**)	20.0 ± 15.2 (5.7)
	Pb	54.9 ± 32.5	2.3 ± 2.4 (4.0)	13.8 ± 12.5 (16.8)	55.7 ± 74.4 (42.8)	36.5 ± 26.5 (38.2)	2.3 ± 2.4 (4.0)	10.5 ± 14.9 (**15.4**)	3.5 ± 4.8 (5.7)
2012	Fe	31947 ± 4899	744 ± 887 (2.2)	2708 ± 2261 (9.0)	3841 ± 3029 (13.9)	23877 ± 8957 (74.8)	744 ± 887 (2.2)	1187 ± 913 (**3.6**)	1036 ± 708 (3.1)
	Mn	595 ± 259	214 ± 88 (39.0)	144 ± 186 (20.6)	61.2 ± 39.0 (10.1)	171 ± 148 (29.2)	214 ± 88 (**39.0**)	178 ± 123 (29.2)	135 ± 119 (20.4)
	Zn	2549 ± 1958	720 ± 638 (25.7)	952 ± 1033 (31.8)	579 ± 650 (27.8)	259 ± 424 (14.8)	720 ± 638 (**25.7**)	484 ± 300 (20.1)	132 ± 88 (5.7)
	Cu	188 ± 139	0.30 ± 0.34 (0.60)	4.6 ± 9.2 (4.4)	112 ± 112 (71.1)	20.2 ± 8.8 (24.0)	0.30 ± 0.34 (0.60)	4.4 ± 3.7 (5.1)	14.9 ± 15.0 (**9.3**)
	Ni	86.9 ± 52.2	31.0 ± 33.1 (27.8)	12.4 ± 11.2 (12.5)	14.4 ± 1.6 (22.1)	31.7 ± 21.9 (38.9)	31.0 ± 33.1 (27.8)	44.1 ± 36.7 (**42.7**)	33.9 ± 32.9 (34.4)
	Cr	261 ± 191	6.4 ± 6.1 (2.1)	27.5 ± 35.7 (6.6)	190 ± 185 (54.3)	75.6 ± 74.9 (37.2)	6.4 ± 6.1 (2.1)	36.9 ± 38.1 (**10.6**)	21.8 ± 19.7 (6.6)
	Pb	73.3 ± 31.1	1.8 ± 1.6 (2.3)	17.0 ± 21.9 (20.4)	31.4 ± 20.7 (38.4)	28.5 ± 13.8 (39.2)	1.8 ± 1.6 (2.3)	14.2 ± 27.5 (**14.3**)	9.2 ± 10.9 (10.8)

The influence of pH on metal bioaccessibilities in sediments is coherent with other studies involving sediments (Waziri and Andrews, 2013; Ren et al., 2015) and other matrixes like soils (Poggio et al., 2009; Li and Zhang, 2013), household dust (Turner, 2011) or boat paint particles (Turner and Ip, 2007), in which most metal bioaccessibilities were reduced with pH increasing from gastric to intestinal phase. In the carbonate-rich environment of the intestine phase metals may be stabilized in solution by complexation, undergo re-adsorption to pre-existent or altered sites at the particle surface, or precipitate as relatively insoluble compounds (Ren et al., 2015). For example, Pb becomes less soluble due to complexation with organic matter, sorption on oxide and silicate clay minerals or hydroxides with very limited solubility (Gonnelli and Renella, 2013), and Cr can be adsorbed on formed iron oxides, precipitates as Cr^{3+} or co-precipitates with Fe (Yu, 2012). According to the abovementioned studies, re-adsorption of Cu-complexes is favoured in an acidic environment of gastric phase, while at pH = 7 Cu is mainly stabilized by solubilisation as carbonates and, therefore, its bioaccessibility increased in the intestinal phase.

Regarding the spatial-temporal evolution of metal bioaccessibilities (Fig. 5.3), the sampling sites where the impact of urban and industrial activity was notable or maximum presented the highest bioaccessibilities for all metals (except Cu) in both methods. In contrast, while TCLP showed a significant temporal reduction for almost all metal bioaccessibilities along the catchment, both phases of PBET did not presented a clear decreasing trend of metal bioaccessibilities. In order to establish relations between elements, hence to improve our understanding on the methodology used for bioaccessibility assessment in this study and explain how different parameters influence bioaccessibility spatial-temporal evolution, principal component analysis was performed with the results for metal bioaccessibilities (Table 5.2), pH and Eh of water, and TOC, TN and TS of sediments. In addition, the bioaccessibilities measured with TCLP and PBET were compared with sequentially extracted fractions of BCR using the Spearman correlation analysis. However, instead of considering all metal fractions, only the most mobile fractions were taken into account (F_1, F_2, F_3, F_{12} (F_1+F_2) and F_{123} (F_1+F_2+F_3)).

With regard to TCLP, on the factorial plane I-II (Fig. 5.4A), Factor I accounts for 43.4% of the variance while Factor II accounts for 23.1%. Two groups may be easily

visualized, grouping Eh, pH and Cu bioaccessibility on the one hand and Fe, Ni, Cr, Zn, and Pb bioaccessibilities on the other. Factor I, therefore, represents the intensive interactions between Cu sorption on river sediments and the changing river water parameters (pH, Eh), indicating that at alkaline/oxidizable environments more mobile Cu-binding form in sediment is expected. Liu and Zhao (2007) also found strong influence of pH and Eh on Cu leachability, suggesting the sediment acidity was responsible for the higher Cu leaching. Otherwise, Factor II evidences Ni, Cr, Pb and Zn as the elements associated with Fe solubilisation, as was also observed by Devesa-Rey et al. (2010). Indeed, for these metals, the bioaccessibilities measured by TCLP had a significant positive correlation (> 0.839, $\rho < 0.01$) with the exchangeable-reducible fraction (F_{12}), implying that Fe oxyhydroxides are one of the main sources of their bioaccessible fraction as well as exchangeable and carbonate-bound phase. The sampling points with higher bioaccessible metal percentages (Fe, Ni, Zn, Pb and Cr) can be seen to be distributed towards the positive side of Factor I, progressively followed, in decreasing order of bioaccessible metal content, by D4, E1, D3, O1, M1 and D2. Actually, mid- (D3 and O1) and downstream (E1 and D4) sampling sites were characterized by a higher proportion of Fe, Zn, Ni, Cr and Pb in the exchangeable/carbonates and reducible fractions (F_{12}) (Martínez-Santos et al., 2015). Additionally, due to a slight temporal reduction of almost all metals bound to these fractions (Table 5.2), at all sampling points, except D3 (for Fe and Mn) and D4 (for Cu and Cr), there were strong variations in the bioaccessibility of metals between the two years (Fig. 5.3).

For the gastric phase of PBET, the factorial plane I-II (Fig. 5.4B) reflects the most important information, with Factor I (35.5% of the variance) positively characterised by Zn, Cr and Fe bioaccessibilities, and negatively by pH and Cu bioaccessibility. Mn (29.2%, Table 5.2) and Zn (20.1%, Table 5.2) gastric bioaccessibilities, despite showing a good relationship with F_{12} fractions (> 0.678, $\rho < 0.01$), were lower than the extraction percentage in F_1 fraction of BCR (44.5-33.5% for Mn and Zn, respectively; Table 5.2), which implies that only weakly sorbed metals susceptible to pH changes were preferentially released into the stomach. However, the gastric bioaccessibility of Fe (5.4%, Table 5.2) was higher than the extracted percentage observed for the F_1 fraction of BCR (3.3 %, Table 5.2) but lower than the observed for the F_2 fraction (9.8%, Table 5.2), which is mainly addressed to solubilize the Fe reducible components.

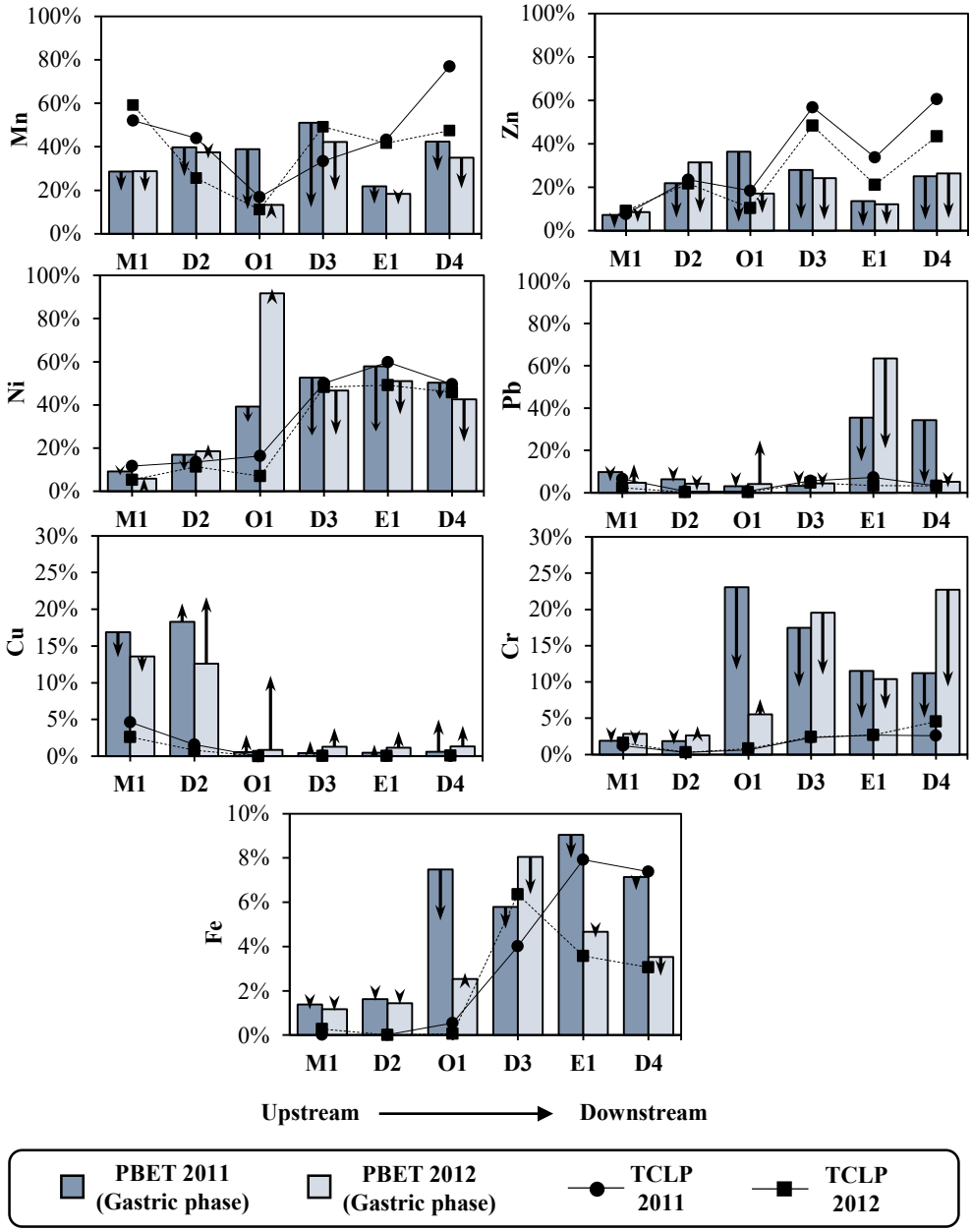

Fig. 5.3. *Metal bioaccessibility spatial evolution for both sampling campaigns and all methods. Bar graphs represent results obtained by gastric phase of PBET, where the arrows indicate the increase/decrease of bioaccessibility in the intestinal phase respect to the gastric phase. Line graphs represent results obtained by TCLP in 2011 and 2012. Note: See different scale for each metal.*

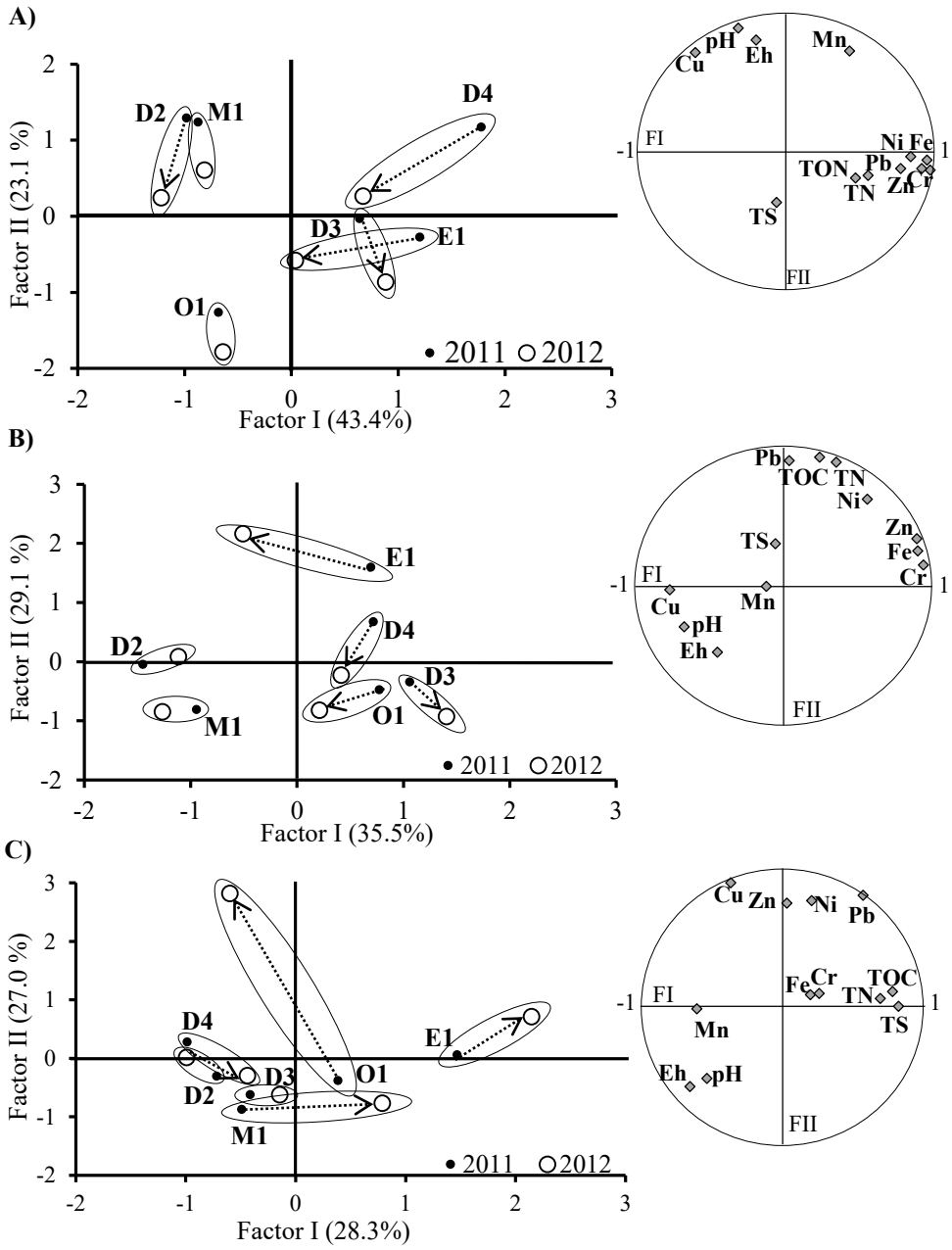

Fig. 5.4. *PCA applied to the results of TCLP (A), gastric PBET (B) and intestinal PBET (C) methods, and physicochemical properties of water and sediments for all sampling sites and both years*

On the other hand, Factor II (29.1%) is characterised by TOC, TN, and Ni and Pb bioaccessibilities. In fact, Ni, Cr and Pb bioaccessibilities have been found to be positively correlated to the oxidizable fraction of the sediment (> 0.790, $\rho < 0.01$), suggesting that organic matter is probably one of the major sinks for the bioaccessible fraction of these metals. The same affinity of gastric bioaccessibilities for organic matter content was observed in previous study on sediments (Devesa-Rey et al., 2010), in which Ni, Cr and Pb bioaccessibilities were linked to fulvic acid, humic acid and particulate organic matter content, respectively. Major influence of organic matter content on Cu gastric bioaccessibility was expected due to its high content in the oxidizable fraction of the sediments (Table 5.2) but the sorption of Cu on solid organic matter and the complexation with dissolved organic matter would result in a small net effect (Degryse, 2009). However, it appeared negatively impacted by Fe/Mn oxide-bound Cu compounds reduction (-0.587, $\rho < 0.05$), which occurs at low pH and Eh values, promoting Cu complexation with dissolved organic matter. Similar results were found by Poggio et al. (2009), in whose study on sediments Cu gastric bioaccessibility was even inversely correlated to organic matter content. The sediment samples with the greatest toxicity risk due to high metal bioaccessibilities (Fe, Ni, Zn, Pb and Cr) and organic matter content are distributed towards the first positive quadrant progressively followed in decreasing order by E1, D4, O1, D3, M1 and D2. Actually, mid- and downstream sites are directly (E1 and O1) or indirectly (D3 and D4) impacted by untreated or partially treated wastewater inputs which contain organic-rich material. In Fig. 5.3, one sampling point in particular shows a strong gastric bioaccessibilities variation from 2011 to 2012. Indeed, the proportion of all metals, except Ni, diminished in the mobile fractions (F_{123}) and increased in the residual phase in 2012 (Martínez-Santos et al., 2015).

Finally, for the intestinal phase of PBET no clear associations were found among metal bioaccessibilities and physicochemical properties of water and sediments (Fig. 5.4C). No major changes in Spearman´s correlation were observed compared to the gastric phase, except for Pb, which does not show any correlation to sequentially extractable fractions. Actually, pH levels in BCR and gastric PBET are acidic while weakly alkaline in intestinal PBET, therefore low pH can help metals to release from the weak binding sites or Fe/Mn sites (Ren et al., 2015). To understand the differences in the pH effect on metal bioaccessibility in the intestinal phase between sampling sites and years, it is important to appreciate that

metal release in a given matrix is controlled by a number of physiological-chemical variables (Turner and Radford, 2010). For example, in subsequently neutralizing the stomach phase to mimic the intestine, different quantities of sodium bicarbonate were required to be added for each SBS sample, to adjust the pH to 7.0, and it is not possible to discriminate the effects of carbonate ion concentration on metal release. In addition, compositional heterogeneities must also be taken into account. For example, all sites showed TS/TOC ratios < 0.2, except M1 in 2012 (= 0.5; Martínez-Santos et al., 2015), suggesting periods of anoxic conditions. This ratio and the high sulphur content of this site, attributed to the presence of anhydrite and gypsum intercalations with limestone and sandstones (Wealden Facies evaporites) (Martínez-Santos, et al., 2015), suggest the precipitation of sulphides like galena (PbS), which contains less bioaccessible Pb than metal-lead oxides or cerussite ($PbCO_3$) (Ruby et al., 1996). With the addition of bicarbonate, lead could be redistributed, benefitting $PbCO_3$ formation, leading to a greater release of lead in the intestinal phase than in the gastric phase. However, Cu presents lower bioaccessibility with pH increasing in M1 because it is highly insoluble in reduced environments, where it precipitates as metal or as very stable sulphides (Du Laing et al., 2007; Weber et al., 2009).

5.4.2. PBET method evaluation

In vitro methods are widely used for estimating bioaccessibility in risk assessment studies, especially in soils, as they represent an ethical, faster and less costly alternative to *in vivo* analysis (Harmsen, 2007; ISO 17402:2008). However, despite numerous procedures have been proposed and reviewed by numerous authors to achieve broadly similar goals, there is lacking of comparison of the extraction efficiencies of sediment-bound contaminants by chemical and biological reagents. In Fig. 5.5, the results of the bioaccessibility assessment evaluation ratio (BAER) for all metals have demonstrated an appropriate employment of biological or enzymatic reagents to simulate gastric and intestinal conditions, since PBET provides higher bioaccessibilities than TCLP ($Log_{10}BAER > 0$), which seems to underestimate the toxicity risk for direct ingestion of sediments. Taking this threshold as a reference, for the gastric phase it can be clearly seen from this plot that more than 50% of $Log_{10}BAER$ values of Mn and Zn were below this limit. In contrast, 75-100% of $Log_{10}BAER$ values for Fe, Cu, Ni, Cr and Pb exceeded the threshold where the median values reflected that PBET results

were one to ten times higher than TCLP results. As regards PBET phase change, a decrease in BAER values was observed for all metals except Cu, between gastric and intestinal phases. For the intestinal phase, once again more than 50% of Log_{10}BAER values for Mn, Zn –and in this phase also for Ni– were below 0.

The difference in results can be primarily attributed to the variation in chemical compositions of digestion matrix, eventually leading to an acidic pH for PBET (gastric phase), which could be expected to favour the mobilization of certain metals from the stomach to the intestine. Moreover, the addition of organic acids such as acetic, citric, lactic, and malic acid can increase the bioaccessibility of certain metals (Yu, 2012) and enzymes can break down the organic polymers into smaller molecules that can be absorbed easily by the human organism, so metals, particularly, those adsorbed to organic matter, are more easily extracted in PBET than in TCLP (Turner and Olsen, 2000).

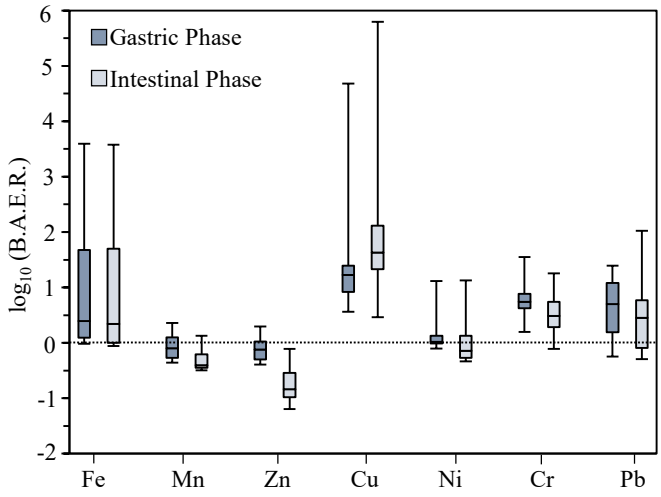

Fig. 5.5. *Boxplot representation of Log_{10}BAER ratio for all metals and both PBET phases. Bioaccessibility data from two sampling years have been considered (N =12). The line is the limit to consider PBET as good bioavailability predictor (Log_{10}BAER > 0)*

An analysis of variance (ANOVA) was performed to study whether values of metal bioaccessibilities were dependent upon the type of analysis method. Significant interactions ($\rho > 0.05$) between TCLP and PBET (both phases) were found for all metals except for Fe (in M1 and D2), Mn (in M1 and E1), Zn (in M1, D3, E1 and D4), Cu (in M1, D2 and D4), Ni

(in D3), and Cr (in D3 and E1). In general, upstream (M1 and D2) sampling points showed considerable differences between methods for Fe, Mn, Zn and Cu. Nevertheless, mid-(D3) and downstream (E1 and D4) sampling points exhibited a great disparity between methods for Mn, Zn, Cu, Ni and Cr. On the other hand, major interactions between gastric and intestinal phases were found for all metals except for Zn (in D2 and D3), Cu (in D4), Ni (in D3), and Cr (in D3 and E1).

5.4.3. Health assessment

Rather than considering each metal HQ index individually, the sum of all HQ values for each SBS samples from two sampling campaigns and for each phase of the PBET method is taken into account in Fig. 5.6A. In general, the hazard quotient was higher for the gastric phase than for the intestinal phase, except at M1 and, especially at O1 in 2012. As regards temporal changes, a decrease (5-48%) in gastric HQ index was detected for all SBS samples, except D2 and D3 (18-26% of increase), while all SBS samples presented an increase of HQ index for the intestinal phase (11-31%), except D4 (24% of reduction).

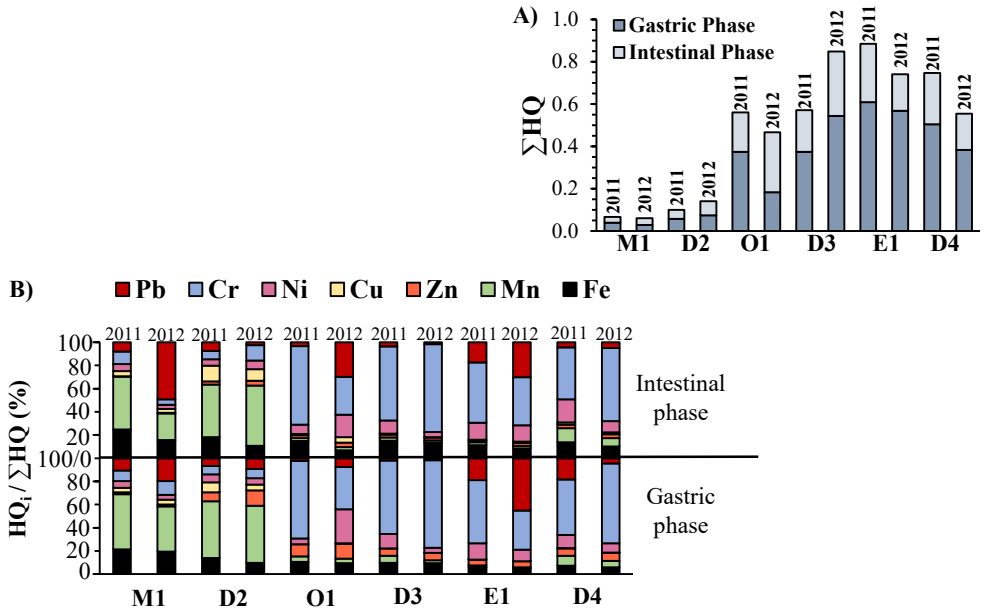

Fig. 5.6. *HQ index in gastric and intestinal phases for each sampling site and both sampling years. A) all metals have been considered (∑HQ); B) the contribution of each metal in the sum of all HQ is considered (HQ_i/∑HQ (%))*

The same metal toxicity pattern was repeated for both years in fine sediments from the Deba catchment, meaning that E1 was the most hazardous sampling point followed by D4 > O1 > D3 > D2 > M1, except in 2012 where D3 > D4. Although high HQ index values (> 1) were not observed, the middle and lower part of the catchment might be considered the most hazardous locations for children. Finally, Fig. 5.6B shows the contribution of each metal in the sum of all HQ values for each site from two sampling years and for each phase of the PBET. Mn, Ni and Zn were the most bioaccessible metals due to their high extent in the exchangeable/carbonates (F_1) and the reducible (F_2) fractions, and consequently, their susceptibility to remobilization if the physicochemical conditions (e.g., pH, Eh, etc.) of the river change is higher (Sakan et al., 2009). However, because of its considerably low oral reference doses, Cr seemed to be the most potentially toxic element downstream while Mn had high potential risk to cause harmful effects upstream, except at M1 in 2012, where Pb became the most dangerous metal (Fig. 5.6).

5.5. Conclusions

The introduction of bioaccessibility into human health risk assessment is becoming increasingly common for a more realistic estimation of the capacity of pollutants to be incorporated into the food chain. However, despite acting as storage and transport vectors, investigations on human bioaccessible fraction of sediment-bound contaminants are still insufficient and a wider variety of studies are needed. This study, focuses on a comparison of various *in vitro* analysis methods proposed by different authors and guidelines, demonstrates that the bioaccessibility of metal bound to Deba River urban catchment sediments was generally higher in the physiological based extraction test (PBET) than in the chemical method (TCLP), implying that acidic environment (pH 2.5) and complexation by organic acids and enzymes play a crucial role on metal release (Fe, Ni, Cu, Cr, and Pb). Instead, when the intestinal phase was simulated (pH 7.0) solubilisation of Cu was enhanced, resulting in bioaccessibility increase.

Although guidelines establish that bioaccessibility may be only estimated from relationships with pseudo-total content, strong influence of water and sediment parameters, such as pH, Eh and organic matter was derived. Further, evaluation of metal bioaccessibility

together with metal fractionation was recommended since metal-binding form affects their mobility. In fact, metal bioaccessibility in gastric and intestinal phases was well correlated with F_1, F_2 and F_3 fractions, which suggests that metals bound to exchangeable and carbonate, Fe–Mn oxide, and organic fractions were preferentially released into the gastrointestinal tract. Finally, untreated and partially treated wastewater inputs, which are considered to be a significant source of organic matter and metals in river sediments, seems to provide more bioaccessible metals and be the main reason for the high human health risk of sediments at mid-(O1 and D3) and low-water locations (E1 and D4), where the majority of metals were associated with the mobile fractions (F_1, F_2 and F_3).

5.6. References

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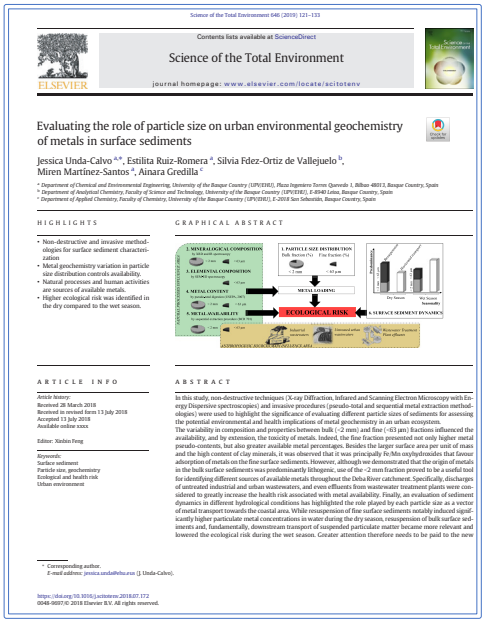
6

Results and discussion II

Evaluating the role of particle size on urban environmental geochemistry of metals in surface sediments



6. Evaluating the role of particle size on urban environmental geochemistry of metals in surface sediments

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6.1. Abstract

In this study, non-destructive techniques (X-ray Diffraction, Infrared and Scanning Electron Microscopy with Energy Dispersive spectroscopies) and invasive procedures (pseudo-total and sequential metal extraction methodologies) were used to highlight the significance of evaluating different particle sizes of sediments for assessing the potential environmental and health implications of metal geochemistry in an urban ecosystem.

The variability in composition and properties between bulk (< 2 mm) and fine (< 63 µm) fractions influenced the availability, and by extension, the toxicity of metals. Indeed, the fine fraction presented not only higher metal pseudo-contents, but also greater available metal percentages. Besides the larger surface area per unit of mass and the high content of clay minerals, it was observed that it was principally Fe/Mn oxyhydroxides that favour adsorption of metals on the fine surface sediments. However, although we demonstrated that the origin of metals in the bulk surface sediments was predominantly lithogenic, use of the < 2 mm fraction proved to be a useful tool for identifying different sources of available metals throughout the Deba River catchment. Specifically, discharges of untreated industrial and urban wastewaters, and even effluents from wastewater treatment plants were considered to greatly increase the health risk associated with metal availability. Finally, an evaluation of sediment dynamics in different hydrological conditions has highlighted the role played by each particle size as a vector of metal transport towards the coastal area. While resuspension of fine surface sediments notably induced significantly higher particulate metal concentrations in water during the dry season, resuspension of bulk surface sediments and, fundamentally, downstream transport of suspended particulate matter became more relevant and lowered the ecological risk during the wet season. Greater attention therefore needs to be paid to the new hydrological scenarios forecast to result from climate change, in which longer seasons with low river discharges are forecast.

6.2. Introduction

According to World Population Prospects (United Nations, 2017 Revision), the world's population is projected to grow from nearly 7.6 billion in 2017 to 8.6 billion in 2030. Intense development of human activity, driven by demographic growth, will greatly impact environmental quality and by extension, human health (Ghanem, 2018). Several studies have addressed the impact of urbanization on the quality of aquatic environments, including high loadings of nutrient and microbial contaminants from both septic systems and wastewater treatment plants (WWTPs) (Carey et al., 2013; McGrane et al., 2014), volatile organic compounds from vehicular emissions (Mahbub et al., 2011) and metals, mainly from industrial or urban effluents (Gupta et al., 2010; Buzier et al., 2011), among others.

Sediment is a good indicator of pollution loads in rivers since it is subject to a continuous accumulation of pollutants, especially metals (Devesa-Rey et al., 2010; Bartoli et al., 2012). These contaminants are considered to pose a serious threat to ecological and human health because of their non-biodegradable, toxic and persistent nature, as well as their capacity to enter the food chain (Burghardt, 1994; van Kamp et al., 2003; Armitage et al., 2007). The percentage of total metal content in the sediment that is available for absorption into the systemic circulation system and that has a toxic impact on human health, will depend firstly on environmental availability (Lanno et al., 2004; Harmsen, 2007), which is in turn related to its chemical forms or types of binding (Saracoglu et al., 2009; Sungur et al., 2014). The physical and geochemical properties of sediments such as surface to volume ratio, mineralogical composition and organic matter are considered to influence chemical distribution of metals (Simpson and Batley, 2009; Campana et al., 2012; Saeedi et al., 2013; Zhang et al., 2014). However, particle size determines all these properties and, therefore, it is the cornerstone parameter (Maslennikova et al., 2012).

To date, numerous researchers have used different analytical techniques (X-ray Diffraction, Infrared and Scanning Electron Microscopy or Inductively Coupled Plasma) to individually investigate the grain size effect over: the mineralogical and elemental composition (Zhou et al., 2015) or the metal chemical speciation (Liang et al., 2018); as well as the mineralogical effect over the metal content (Xie et al., 2018). However, considering all these techniques as a whole may be more suitable to bind the particle size effect on the geochemistry of sediments with the metal accumulation, distribution and environmental impact. In urban environments, geochemical patterns observed in different particle sizes of sediments help to differentiate the contribution to metal availability of non-anthropogenic sources from human activities (Chiprés et al., 2009). Indeed, sediment records the geochemical composition of the provenance bedrock and the intensity of chemical weathering and hydraulic sorting (Lapworth et al., 2012; Zhao and Zheng, 2015; Kirkwood et al., 2016; Darwish, 2017). During chemical weathering of the bedrock, water-soluble elements are chemically dissolved in water, whereas water-insoluble elements are physically transported by the water current (Zhao and Zheng, 2014). Consequently, despite the high adsorption capacity of minerals, the chemical dissolution of soluble ones might promote the availability of metals previously retained in their lattice.

Additionally, after elucidate the source and magnitude of available metals in each particle size, sediment dynamics become decisively important in addressing the ecological consequences of seasonal variations in river discharge. Indeed, the physical processes involved in sediment dynamics include erosion, transport, deposition, and resuspension (ICES, 2011), which are the result of interactions between several variables such as water discharge or grain size distribution (USEPA, 1999; Apitz, 2012). According to future climate change predictions, alterations in the seasonal precipitation (magnitude and duration) and, consequently, river flow variations are expected. Therefore, deeper knowledge into sediment dynamics will be crucial for a better estimation of metal environmental risk, based on the new hydrological scenarios.

The overall aim of this study was therefore to identify the relevance of analysing different particle sizes, as a more reliable reflection of the environmental geochemistry of metals in surface sediments from an urban catchment and the associated ecological and human health risk. The specific objectives were (i) to use different methodologies for mineralogical, elemental and metal characterization of surface sediments, (ii) to identify natural processes and/or sources of anthropogenic contamination influencing the environmental and health risk associated with the availability of metals in surface sediments, and (iii) to evaluate the influence of seasonality on physical mechanisms governing metal migration towards the coastal area. We hypothesized that combined analysis of different particle sizes of surface sediment will provide us more comprehensive and detailed information about the environmental geochemistry of metals in a catchment subjected to multiple pressures.

6.3. Materials and methods

6.3.1. Study area and sampling

The Deba River runs through the catchment (538 km^2) to the Bay of Biscay, receiving inflows from several tributaries, including the Ego and Oñati streams (Fig. 6.1). The geology of the catchment mainly consists of a succession of sedimentary rocks, predominantly an alternation of sandstones and mudstones in the southern and western part, marls in the central area, and limestones in the northern region. In contrast, igneous rocks dominate the area of

confluence of the Ego tributary and the main river (Fig. 6.1A).

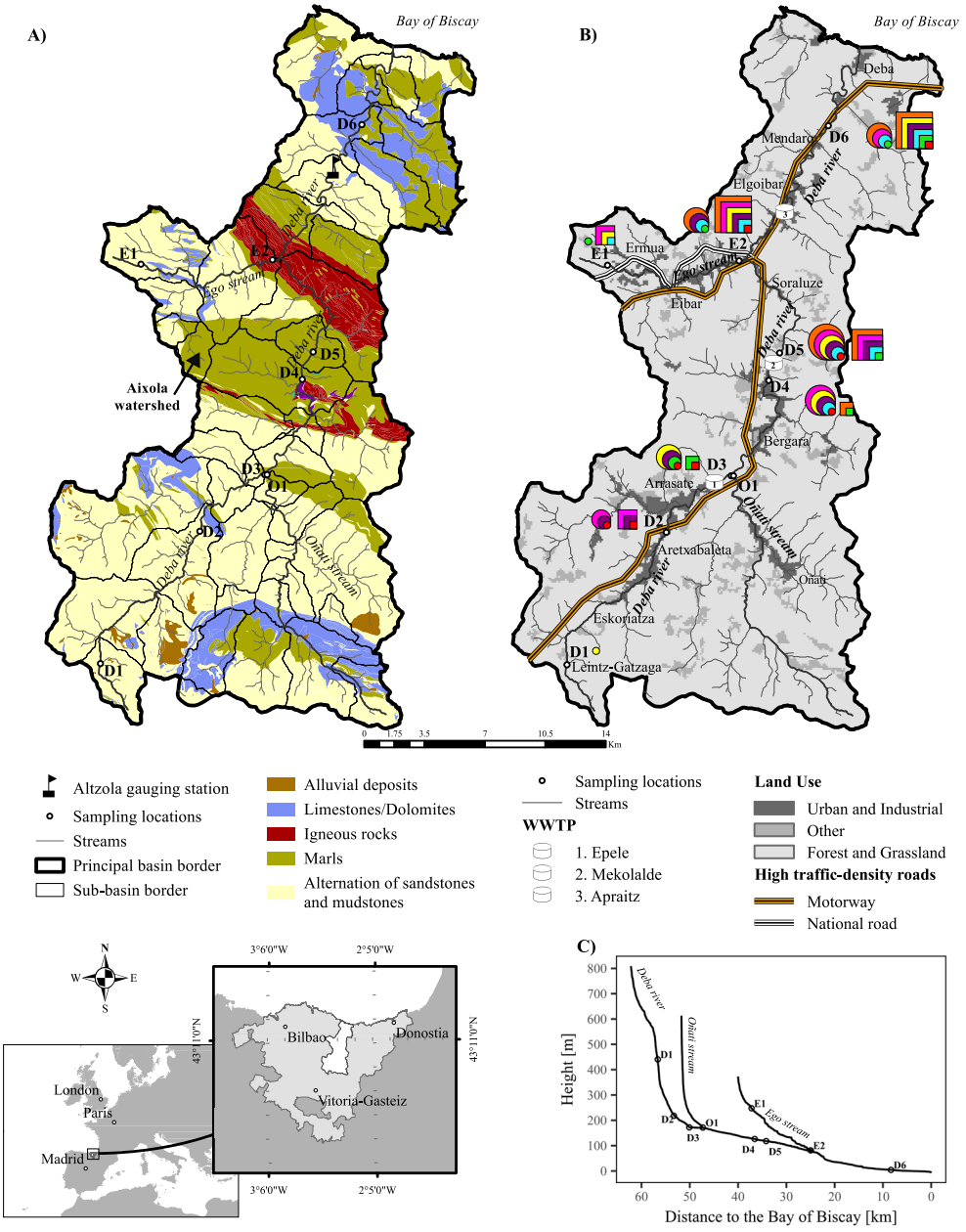

Fig. 6.1. *Location of Deba River catchment, and (A) lithological map, (B) land use map, and (C) height profile. The sub-basin subdivision and the location of Altzola gauging station can be observed in (A). The sampling sites, the wastewater treatment plants (WWTPs) and high traffic-density roads are shown in (B). Circles and squares represent the anomalies (normalized-concentration > 1) shown by each metal (Fe: red; Mn: green; Zn: blue; Cu: purple; Pb: yellow; Cr: pink; Ni: orange) in the bulk and the fine sediments, respectively*

The Deba River catchment possesses certain characteristics which are distinctive of a typical urban environment. These include dense population, a relatively high level of productivity, primarily driven by non-agricultural activities, infrastructures, buildings, and an extensive motor transportation network (Wong et al., 2006; Fig. 6.1B). Surface waters receive treated effluents from three wastewater treatment plants (Fig. 6.1B). The Apraitz (95,000 population equivalent (pe)) and Mekolalde (35,000 pe) WWTPs have been operating since 2007 and 2008, respectively. The Epele WWTP (90,000 pe) only came into continuous operation in May 2012; previously, organic-rich wastewaters from the towns of Arrasate and Oñati were discharged into the Deba River and Oñati stream. In June 2014, the sewer from Ermua-Eibar was also connected to the Apraitz WWTP and untreated urban wastewater (UWW) from these municipalities is no longer discharged into the Ego stream. However, according to data from Gipuzkoa Provincial Council, about 6773 m^3 y^{-1} of untreated industrial wastewater (IWW) from metal-working, the automotive industry, galvanising, smelting factories and electrical appliance manufacturers are also discharged into the Deba River and its tributaries (Martínez-Santos et al., 2015).

In this context, surface sediments are characterized by having a high content of metals, nutrients and organic compounds, principally in areas of greatest urbanization and industrialization. Previous studies have shown that discharges of effluents from WWTPs and even IWW and UWW throughout the catchment are responsible for the declining quality of surface sediments, posing a potential risk for the ecosystem (Unda-Calvo et al., 2019) and for human health (Unda-Calvo et al., 2017). However, no study has addressed the role of sediment particle size in the mobilization, deposition, and dispersion of potentially toxic metals in the Deba River catchment.

In October 2015, surface sediment samples were collected from six sampling sites along the main river bank (D1, D2, D3, D4, D5 and D6) and from two sampling sites in the Ego tributary (E1 and E2) (Fig. 6.1). These sampling locations were chosen with a view to studying the influence of natural processes or anthropogenic metal sources on the chemical quality of surface sediments. As per USEPA (2001), surface sediment subsamples (0-5 cm depth) from multiple points within each sampling site were collected using a sterilized plastic spoon, sieved through a 2 mm mesh, composited in the field and sealed in sterile

polypropylene bags.

A water sample gathering programme was established to study the involvement of suspended particulate matter (SPM) in metal transport towards the Bay of Biscay. The programme consisted of manual sampling of water in sterile polyethylene bottles monthly or bimonthly from January 2015 to January 2016 at the same six sampling sites along the main river and at three sampling sites in the Oñati (O1) and Ego (E1 and E2) tributaries. In addition, discharge data (Q, $m^3 s^{-1}$) were monitored daily at the catchment outlet in the Altzola gauging station (www.gipuzkoahidraulikoak.eus) to determine different hydrological conditions during the study period. All water and sediment samples were stored and refrigerated in the dark and transported to the Chemical and Environmental Engineering laboratory (University of the Basque Country) on the same day.

6.3.2. Laboratory methodology

Surface sediments were air-dried and ground with a pestle and mortar for homogenization. The fine fraction of the surface sediments (< 63 μm) was sieved through a stainless-steel sieve. The moisture content of all samples was determined in accordance with APHA, 2015.

6.3.2.1. Molecular/Mineralogical analysis

For molecular characterization of the surface sediment samples, X-ray Diffraction (XRD) and infrared (IR) spectroscopies were used. The XRD analyses were performed using a PANalytical Xpert PRO powder diffractometer equipped with a copper tube (λCuKαmedia = 1.5418 Å, λCuKα1 = 1.54060 Å, λCuKα2 = 1.54439 Å), a vertical goniometer (Bragg–Brentano geometry), a programmable divergence aperture, an automatic interchange of samples, a secondary graphite monochromator, and a PixCel detector. The software PANalytical X'pert HighScore can provide a semiquantitative approximation of the compounds in each sample. The IR laboratory equipment was a Jasco 6300 FTIR spectrophotometer in transmittance mode. All IR spectra obtained in the laboratory were collected in the middle infrared region (from 4000 to 400 cm^{-1}), recording 32 scans per spectrum at a spectral resolution of 4 cm^{-1}.

6.3.2.2. Elemental analysis

For the elemental spectroscopic analysis, 0.5 g of the fine surface sediment samples was pressed at a pressure of 9 t in a CrushIR (PIKE Technologies, Canada) hydraulic laboratory press; the final pellets had an approximate diameter of 12 mm and an approximate width of 1 mm. For the purposes of creating elemental distribution maps, the < 63 µm grain size sediment samples were selected, due to the higher concentration of the study elements in this fraction.

Scanning Electron Microscopy with Energy Dispersive Spectroscopy (SEM-EDS) analyses on fine sediment pellets was used for electron image acquisitions and elemental composition determination using an X-Max energy dispersive X-ray spectrometer (Oxford Instruments, Abingdon, Oxfordshire, U.K.) coupled to an EVO40 scanning electron microscope (Carl Zeiss NTS GmbH, Germany). SEM images were acquired at high vacuum, employing an acceleration voltage of 20 KV. Magnifications up to 10,000× were achieved using a secondary electron (SE) detector. The elemental analysis was carried out using a working distance of 8.5 mm, take-off angle of 35°, and an acceleration voltage of 30 kV. An integration time of 50 s was used to improve the signal-to-noise ratio of EDS spectra. Spectral data were processed using INCA software (Oxford Instruments, Abingdon, Oxfordshire, U.K.). This software can provide a semiquantitative approximation of the elements contained in the surface sediment samples under study, based on the K-alpha net areas of each element detected. Additional information relating to the instrument, measurement conditions, and spectral assignment can be reviewed elsewhere (Aramendia et al., 2018; Gómez-Nubla et al., 2013).

6.3.2.3. Pseudo total and sequential metal extraction

The pseudo total metal content was measured using an ETHOS 1, Millestone microwave digestion system, where three replicates of each surface sediment sample (0.5 g) were heated in Teflon vessels with concentrated HNO_3:HClO (3:1.5) by raising the temperature to 180 °C for 10 min and maintaining this level for an additional 25 min (USEPA, 2007).

The metal distribution was determined by sequential extraction of metals using the

procedure developed by the European Community Bureau of References (BCR 701), which is divided into four operationally-defined fractions: (i) exchangeable and acid-extractable (F_1, soluble species, carbonates and exchangeable metals), (ii) reducible (F_2, Fe/Mn oxyhydroxides), (iii) oxidizable (F_3, organic matter and sulphides), and (iv) residual fraction (F_4, remaining non-silicate bound metals).

The metals under consideration (Fe, Mn, Zn, Cu, Ni, Cr and Pb) in the sequential extracts and pseudo-total digestion were determined by ICP-OES (Perkin Elmer Optima 2000). The detection limit for these metals was: Pb (1 µg g^{-1}), Zn (0.5 µg g^{-1}), Fe and Mn (0.4 µg g^{-1}) and Cr, Cu and Ni (0.1 µg g^{-1}). Additionally, the recovery percentage for each metal in each step of sequential extraction was calculated taking into account pseudo-total digestion. For all elements, it stood in a range of 94% to 119%. For the purposes of controlling analytical methods, a NBS sediment sample was also used (Buffalo River sediment, USA). Using this technique, all metals were also measured with mean values close to the certified contents and variation coefficients lower than 8%, except for Pb (17% with 0.5 g of sample).

Water samples collected at all sampling locations were filtered through 0.45 µm filters and the residue was oven-dried at 105º for 1 h. The concentration of the SPM was obtained from the weight of each dried residue and the volume of the sample. The pseudo-total metal (Fe, Mn, Zn, Ni, Cu, Cr and Pb) contents in the SPM were determined using the acid-digestion methodology described above for surface sediments.

6.3.3. Assessment of chemical quality of surface sediments

Based on the work of Skeries et al. (2017), it was decided to use the Divergence Factor (DF) in this study to identify how far a sampling location deviated from the dominant trend in the Deba River catchment in terms of pseudo-total content of the metals in surface sediments. Firstly, the pseudo-content of each element from each sampling site [M] was divided by its respective median value throughout the catchment (Eq. (6.1)). Thus, metal content in surface sediments was considered anomalous when normalized concentration exceeded 1. The median-normalized values for the metals of interest were then totalled for each sampling site (Eq. (6.2)).

$$\text{Normalized concentration} = \frac{[M]}{\text{Median } [M]} \tag{6.1}$$

$$DF = \sum_{i=1}^{n=7} \text{Normalized concentration} \tag{6.2}$$

Enrichment of a given element in sediments relative to a background reference site is an indication of the contribution from nature (e.g. weathering process of rocks) and anthropogenic sources (Violintzis et al., 2009; Legorburu et al., 2013; Gu et al., 2015). The Enrichment Factor (EF) for each metal was calculated in accordance with Eq. (6.3), where $[M]_S$ and $[R]_S$ are the concentrations of the metal M and the reference element R in surface sediment samples, while $[M]_{rf}$ and $[R]_{rf}$ are the concentrations in the upper continental crust (Delshab et al., 2017). The use of local non-polluted sediments as the background reference instead of the crust has been widely proposed in order to deal with the geochemical heterogeneity in nature (Reimann and De Caritat, 2005; Dung et al., 2013; Mali et al., 2015). However, we declined to establish the headwater locations (D1 and E1) as the reference due to the unexpectedly high concentrations of some metals with respect to the region as a whole (Table 6.1). Given that the surface sediments from the Deba River catchment were rich in Fe content and that Fe was strongly retained in the lattice of mineral in both bulk (< 2 mm) and fine (< 63 μm) fractions (as shown below), it was considered to be mainly a lithogenic component, and thus an appropriate reference element for EF calculation. Jiao and colleagues (2015) proposed a six-category system to describe the contamination level. EF < 1, no enrichment; 1≤ EF < 2, deficiency to minimal enrichment; 2 ≤ EF <5, moderate enrichment; 5 ≤ EF < 20, significant enrichment; 20 ≤ EF < 40, very high enrichment; and EF ≥ 40, extremely high enrichment. The global enrichment factor (GEF) is equal to the sum of the enrichment factor of all metals at each sampling site (Eq. (6.4)).

$$EF = \frac{([M]_S/[R]_S)}{([M]_{rf}/[R]_{rf})} \tag{6.3}$$

$$GEF = \sum_{i=1}^{n=6} EF_i \tag{6.4}$$

Determination of Individual and Global Contamination Factors of metals (ICF and GCF, respectively) is an important tool for indicating the degree of risk of metal contamination

to the environment in relation to retention time in sediments (Naji et al., 2010; Saleem et al., 2015). ICFs were obtained for each metal from the results of the fractionation study, dividing the sum of the concentrations (C) in the first three extractions (F_1, F_2 and F_3, constituting the non-residual fraction) by that in the residual fraction (F_4) at each sampling site (Eq. (6.5)). The GCF is equal to the sum of individual factors (Ikem et al., 2003; Naji et al., 2010), as shown in Eq. (6.6).

$$\mathrm{ICF} = \frac{\mathrm{C\ non-residual}}{\mathrm{C\ residual}} \tag{6.5}$$

$$\mathrm{GCF} = \sum_{i=1}^{n=7} \mathrm{ICF_i} \tag{6.6}$$

6.3.4. Statistical analysis

Once a Shapiro Wilk test confirmed that variables were not normally distributed, all data were log-transformed in order to reduce the skewness of the data.

A Spearman correlation analysis (non-parametric test) was performed with metal pseudo-contents to establish the relationships between the bulk and the fine surface sediments. In addition, the effect of variability of composition and properties between particle sizes on the availability of metals in surface sediments was analysed using one-way ANOVA (taking $\rho < 0.05$ as significant, in accordance with Tukey's multiple range test). The variability of metal pseudo-contents in SPM during different hydrological conditions was also analysed to evaluate the influence of seasonality on sediment dynamics. In addition, the predominance of two physical mechanisms involved in metal dispersion were evaluated using regression analysis between surface sediments and SPM. Finally, principal component analysis (PCA) was used to identify the natural process or anthropogenic activity representing the main source of available metals in surface sediments at each sampling site. PCA with an eigenvalue of over 1 was subjected to an orthogonal varimax rotation. This maximises the variance to obtain a pattern of loadings for each factor that is as diverse as possible, thus making it easier to interpret. Statistical processing of the data was performed using SPSS 22.0 software.

6.4. Results and Discussion

6.4.1. Mineralogical and elemental characterization of surface sediments

XRD analyses were performed for molecular and mineralogical characterization of the surface sediment samples. The diffractograms (see for example Fig. S6.1) showed quartz (SiO_2), calcite ($CaCO_3$, except in D1), illite ($K_{0.65}Al_{2.0}[Al_{0.65}Si_{3.35}O_{10}](OH)_2$), and clinochlore ($Mg_5Al(AlSi_3O_{10})(OH)_8$) in all samples and both grain sizes. Albite ($NaAlSi_3O_8$) was observed particularly at D6 (< 63 µm). Oxides and hydroxides of iron (hematite, Fe_2O_3 and goethite, FeO(OH)) were found as trace, except in the case of E2 (< 2 mm) for hematite.

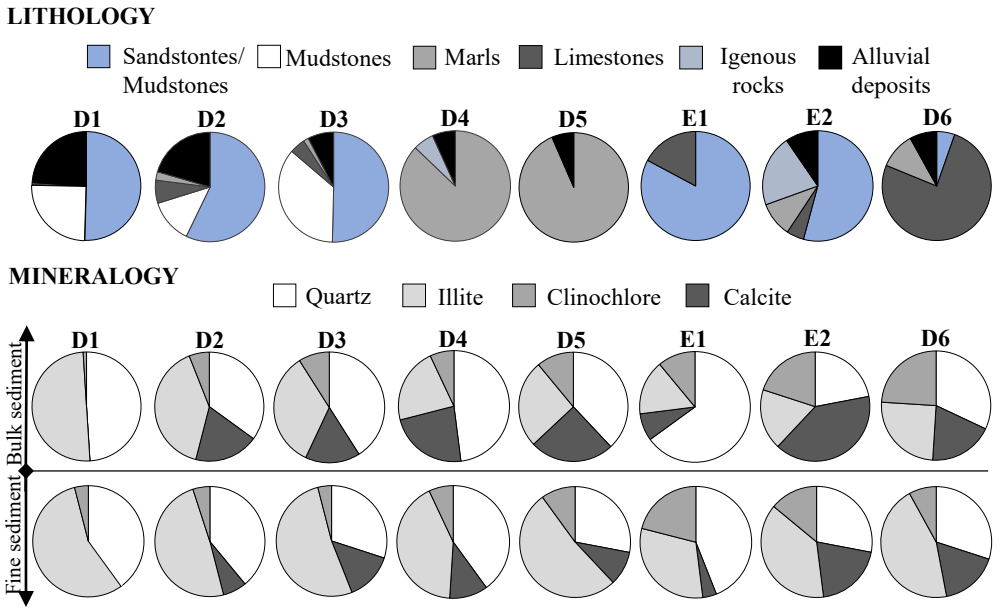

Fig. 6.2. *Lithology of the sub-basins where sampling sites are located and percentage mineralogical distribution of the bulk and fine sediment samples using XRD*

As an estimation of the relative proportion of the identified minerals, the semiquantitative results (Fig. 6.2) showed that all fine (< 63 µm) surface sediments, except D1 and E1 had the following mineralogical composition order: Illite > Quartz > Calcite > Clinochlore. In contrast, the compositional sequence of bulk (< 2 mm) surface sediments varied throughout the catchment, suggesting that the bulk fraction better integrates and explains the differences in the mineralogical composition of the study area, whereas the fine fraction is useful for evaluating the extent and influence of weathering on the maturity of surface sediments. The

term maturity refers to the cumulative changes undergone by sedimentary particles during erosion, weathering, and transport until their final deposition as sediments (Warrier et al., 2016).

Thus, given that carbonate minerals have higher solubility than silicate minerals (Szramek et al., 2011) and smaller particles have a larger surface area in contact with water, we expected to find lower percentages of unstable ones (e.g. calcite) in the fine surface sediments than in the bulk surface sediments. In effect, the higher the particle-size, the greater the proportion of quartz (except at D2 and E2), clinochlore (except at D1 and E1) and, primarily, calcite. Conversely, the percentage of illite, a clay mineral and one of the products of feldspar dissolution (Ma et al., 2017), was higher in the fine surface sediments. In addition, the presence of carbonate minerals in the bulk mineral analysis of the loess/palaeosol sequence was used by Terhorst and colleagues (2012) to classify the weathering intensity at the lower degree. Based on that classification, surface sediments from the Deba catchment would be considered to be in the earliest stages of maturity. Indeed, water erosion is the dominant geomorphological force ahead of karstification in calcareous sites, due to the high rainfall and orographic features of the region (URA, 2004).

Focusing on the bulk fraction, the spatial distribution of the mineralogy concurs with the lithological characteristics of each sub-basin (Fig. 6.2). The headwater of the main channel (D1) was characterized by high proportions of illite and quartz, and the absence of calcite, due to the predominance of sandstones/mudstones and the limited occurrence of limestones in the area. D2-D3 and D4-D5 presented similar lithology and, therefore, mineralogy. In contrast to D1, an increase in the occurrence of limestones and marls, especially at D4 and D5 (Fig. 6.2), encouraged higher calcite proportions downstream. The highest percentages of quartz were observed upstream of the Ego tributary (E1) due to the predominance of sandstones/mudstones, while downstream (E2) calcite was the principal mineral. The fact that carbonate rocks are not characteristic of the lithology at E2 suggests that this mineral was transported from the Aixola watershed (Fig. 6.1A), where most of the main bedrock consists of practically impervious Upper Cretaceous Calcareous Flysch (Meaurio et al., 2015). Finally, despite the fact that limestones predominate in the lithology, the outlet of the catchment (D6) presented similar percentages of all minerals. This could be attributed to the confluence of the Deba

River with the Ego tributary, which considerably increases the river discharge. Hence, a greater discharge explains extensive transport of silicate minerals caused by water erosion of the sandstones/mudstones that form the bedrock from immediately upstream (Altzola gauging station sub-basin, Fig. 6.1A).

In accordance with the XRD results, the mid-infrared (MIR) spectra (Fig. S6.2) identified O—H stretching of free and bound hydroxyl groups (at 3628 cm^{-1}), suggesting the presence of clay minerals. The detected Si—O—Si and Si—O stretching vibrations (at 1000 and 694 cm^{-1}, respectively) may be related with silicate minerals —principally illite and kaolinite—, while quartz double peaks are observed at 776 and 795 cm^{-1}. Finally, the bands located at 1420, 872 and 712 cm^{-1} are associated with the presence of calcium carbonate, and weak peaks at around 796 cm^{-1}, 745 cm^{-1} and 725 cm^{-1} could be attributed to other carbonated elements (e.g. Mg^{2+}, Li^{+}, K^{+} and Na^{+}) (Song et al., 2012). Moreover, the curvature found at 912 cm^{-1} may be characteristic of Al—OH stretching vibration due to the presence of $Al(OH)_3$ or even hematite. Broad bands corresponding to C—H (from 200 to 3100 cm^{-1}) and C=O (at 1797 cm^{-1}) are likely to be characteristic stretching vibrations of aliphatic hydrocarbons, and aldehyde, ketone, ester or carboxylic acid, respectively. On the other hand, the band at 1636 cm^{-1} corresponds to C=C and C=O characteristics stretching vibration of organic matter. As for spatial distribution, it should be highlighted that both particle sizes of surface sediments presented higher areas of these bands downstream of all the WWTPs (D3, D5 and D6).

For elemental characterisation of the fine surface sediment samples, we selected different areas on each pellet where Fe, Mn, Cu, Ni, Cr, or Zn had previously been identified in several EDS spectra. Firstly, the omnipresence of illite in the fine surface sediments (Fig. 6.2) was corroborated since the similarity among K, Al and Si maps implied the formation of potassium aluminium silicates (Fig. S6.3A). Mg also appeared with Al and Si, indicating the presence of magnesium aluminate or magnesium alumina-silicate as clinochlore (Fig. S6.3B). On the other hand, no correlation among Ti, Ca, Fe and Mg maps (Fig. S6.3A) suggested the presence of oxides such as rutile or anatase (TiO_2), hematite (Fe_2O_3) or goethite (FeOOH). Finally, a high correlation of Fe with Al and Si, and with K^{+}, Na^{+} or Mg^{2+} highlighted that it was preferentially retained in the mineral lattice of fine surface sediments. Alternatively, the distribution maps of Zn, Cu, Ni, and Cr showed no correlation with other elements except O

and C. This might indicate that they were presented mainly as oxide or hydroxide or bound to the organic matter, except at D6 and E2 where Cu and Zn, respectively, were correlated with sulphur, suggesting that they were presented as sulphate or sulphide.

6.4.2. Pseudo total and sequential metal extraction in surface sediments

As shown in Table 6.1, median pseudo-contents of metals (Fe, Mn, Zn, Ni, Cu, Cr and Pb) in the fine surface sediments were higher than in the bulk surface sediments, except for Fe and Mn. These results concur with those of other authors who showed that concentrations of metals tend to increase with a decrease in particle grain size (Zhang et al., 2002; Morelli et al., 2012; Yao et al., 2016; Kang et al., 2017). The fine fraction is the most chemically active sediment phase due to its high capacity for cationic exchange, resulting from a high content of secondary minerals (e.g. clay minerals, Fe and Mn oxides and hydroxides, and carbonates) and organic matter (Hardy and Cornu, 2006), and the large surface area per unit mass, which gives it greater adsorption capacity (Guven and Akinci, 2013). Indeed, the bulk fraction had a higher content of quartz, which gives it a very weak adsorption capacity at a particle size of < 2 mm (Horowitz, 1991). Additionally, as observed in EDS spectra of the fine sediments from almost all sampling sites (Fig. S6.3B), Fe and Mn appeared together, suggesting the presence of Fe/Mn oxyhydroxides. However, high quantities of Fe oxides might also be found in the coarse fraction (Devesa-Rey et al., 2011), such as hematite (Fe_2O_3) in the bulk surface sediments from E2. Thus, coatings, probably formed by Fe and Mn oxides on the sandy fraction might explain the predominance of these metals in the bulk surface sediments.

Reflecting the influence of particle size on the adsorption capacity of surface sediments, the metal concentration in the two fractions is not correlated ($\rho < 0.5$) and two different content patterns could be identified: Fe > Mn > Zn > Cu > Cr > Ni > Pb and Fe > Zn > Mn > Cr > Cu > Pb > Ni, for the bulk and fine fractions, respectively (Table 6.1). Otherwise, Ni significantly correlates with Zn ($\rho = 0.976$ at 0.01 level), and in general, positive correlations between Zn, Ni and Cr were found in the bulk fraction. Similarly, these metals are highly correlated in the fine surface sediments, suggesting that they had the same origin. However, correlations between Fe-Cu, Ni-Cu and Pb-Cr ($\rho > 0.714$ at 0.05 level) in the fine fraction imply different possible metal sources.

Table 6.1. *Pseudo-total metal (Fe (mg g^{-1}), Mn, Cu, Cr, Ni, Pb and Zn (µg g^{-1})) content, Enrichment Factor (EF) and Individual Contamination Factor (ICF) were determined in the bulk (< 2 mm) and the fine (< 63 µm) surface sediments at each sampling site in October 2015. Median and standard deviation (± SD) are calculated for each particle size*

		Fe		Mn		Zn		Cu		Ni		Pb		Cr	
	River Sites	*Bulk*	*Fine*	*Bulk*	*Fine*	*Bulk*	*Fine*	*Bulk*	*Fine*	*Bulk*	*Fine*	*Bulk*	*Fine*	*Bulk*	*Fine*
Pseudo-total content	Deba D1	32.9	24.16	419	356	15.5	25.5	26.6	30.5	21.6	36.6	18.6	34.8	170	176
	Deba D2	59.2	30.07	544	491	59.6	100	32.0	35.0	51.5	48.8	12.5	53.2	261	389
	Deba D3	68.2	28.25	972	1177	60.7	70.2	37.4	42.5	28.7	46.5	16.2	29.4	288	421
	Deba D4	68,1	27.22	811	588	58.9	87.8	45.9	53.9	44.0	77.6	26.7	33.1	373	570
	Deba D5	70.8	26.14	902	664	66.1	95.0	46.8	52.8	63.9	78.5	61.8	33.7	407	591
	Deba D6	46.9	37.85	993	1168	51.3	145	68.0	60.4	56.9	88.6	10.3	57.2	406	581
	Ego E1	26.5	22.72	925	442	13.3	44.9	18.5	47.2	33.5	257	13.8	144	148	1087
	Ego E2	34.7	29.77	925	441	69.6	236	38.7	98.7	41.5	318	9.66	101	306	2240
	Median	53.1	27.8	914	539	59.3	91.4	38.1	64.5	42.7	78.0	15.0	44.0	297	576
	% SD	35.3	16.4	26.3	49.1	45.0	65.2	38.3	40.1	33.8	89.9	81.7	67.4	33.8	33.8
EF	Deba D1			0.75	0.86	1.02	2.28	1.34	2.10	0.58	1.34	1.03	2.62	3.07	4.33
	Deba D2			0.54	0.96	2.18	7.20	0.90	1.93	0.77	1.43	0.38	3.22	2.61	7.70
	Deba D3			0.84	2.44	1.92	5.37	0.91	2.50	0.37	1.45	0.43	1.89	2.51	8.85
	Deba D4			0.70	1.27	1.87	6.97	1.12	3.29	0.57	2.52	0.71	2.21	3.25	12.4
	Deba D5			0.75	1.49	2.02	7.85	1.10	3.36	0.80	2.65	1.59	2.35	3.42	13.4
	Deba D6			1.24	1.81	2.36	8.27	2.41	2.65	1.07	2.07	0.40	2.74	5.15	9.12
	Ego E1			2.05	1.14	1.08	4.27	1.16	3.45	1.12	9.98	0.95	11.5	3.32	28.4
	Ego E2			1.56	0.87	4.33	17.1	1.85	5.51	1.06	9.44	0.51	6.16	5.23	44.7
	Median			1.05	1.35	2.10	7.41	1.35	3.10	0.80	3.86	0.75	4.09	3.57	16.1
	% SD			49.6	40.4	48.8	59.3	38.9	36.5	34.6	94.5	56.2	80.4	29.4	84.5
ICF	Deba D1	0.10	0.37	6.63	7.99	0.35	1.54	1.40	1.80	0.23	0.09	3.89	(*)	0.38	1.55
	Deba D2	0.04	0.32	0.96	6.24	0.74	4.59	0.30	1.55	0.19	0.22	2.50	(*)	0.74	4.24
	Deba D3	0.02	0.45	1.74	16.5	0.30	4.31	0.35	2.19	0.11	0.24	0.32	(*)	0.10	5.75
	Deba D4	0.03	0.28	0.82	6.10	0.74	4.34	0.67	2.39	0.87	1.06	1.90	(*)	0.92	7.76
	Deba D5	0.02	0.23	0.74	7.27	0.22	4.97	0.26	2.39	0.36	1.03	0.60	(*)	0.68	8.55
	Deba D6	0.03	0.31	0.69	7.74	0.37	8.13	0.26	2.56	0.16	0.83	0.97	(*)	0.86	7.57
	Ego E1							(**)							
	Ego E2	0.05	0.24	0.30	4.46	0.79	4.74	0.10	2.48	0.32	1.89	3.14	28.6	1.77	9.48
	Median	0.04	0.31	1.69	8.04	0.50	4.66	0.48	2.20	0.32	0.77	1.90	28.6	0.91	6.41
	% SD	66.5	24.4	131	48.7	48.4	41.2	93.0	17.3	80.8	83.7	70.7		47.4	43.2

(*) Pb content in residual fraction is below the detection limit, so that ICF was not calculated. (**) Metal speciation at E1 was not determined, so that ICF was not calculated.

The textures of surface sediments from the Deba River catchment display a predominance of the sandy fraction (0.063-2 mm), with percentages of over 91%. The proportion of heavy metal loading from the fine (< 63 µm) to the bulk fraction (< 2 mm) was calculated from the metal content and the mass percentage of the fine sediment fraction. Only approximately 3.0-14.5 (%) of the total metal loading (not included data) was retained in the < 63 µm fraction, conclusively demonstrating the importance of analysing both particle sizes to provide a more reliable reflection of the risk associated with metals in surface sediments.

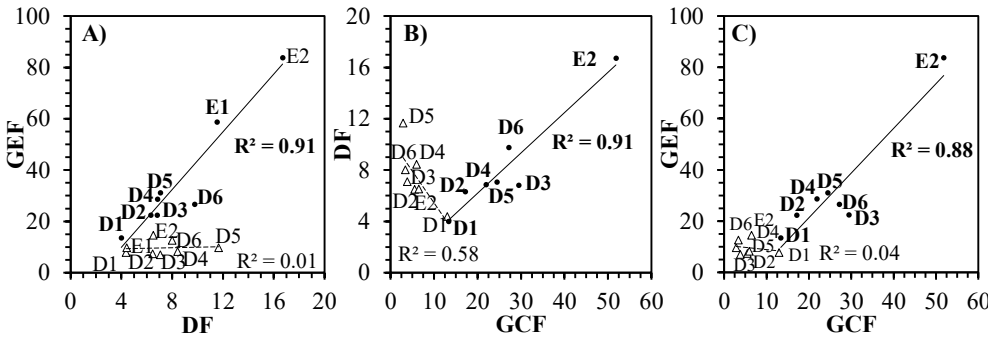

Fig. 6.3. *Linear relationships between Divergence Factor (DF), Global Enrichment Factor (GEF) and Global Contamination Factor (GCF) taking all sampling sites into consideration (except E1 in graphics B and C) for the bulk (< 2 mm; triangles) and the fine (< 63 µm; circles) surface sediments*

As for spatial distribution, the lowest pseudo-contents in the main channel were measured upstream at D1 for all metals, except for Pb (Table 6.1). In contrast, the headwater of the Ego tributary (E1) presented a deviation of over 30% with respect to D1 for Mn, Ni and Cr in the bulk surface sediments, and Zn, Ni, Cu, Pb and Cr in the fine surface sediments, supporting the idea that sampling sites considered as non-disturbed (Fig. 6.2) had a different lithological composition.

By determining the Divergence Factor (DF), it is possible to identify the sampling locations that are most anomalous with respect to the region as a whole, with regard to metal pseudo-content in surface sediments. When the bulk fraction is considered, D4, D5 and D6 are of concern (Fig. 6.3A). Indeed, D5 and D6 had the highest concentrations of Fe-Cr-Pb and Mn-Ni, respectively (Table 6.1). However, the maximum values for Pb, and for Cu, Ni, Cr and Zn were found in the fine surface sediments up- (E1) and downstream of the municipalities

of Ermua and Eibar (E2) respectively. Consequently, the Ego tributary, and the mid-part and the outlet of the Deba River are *a priori* the most potentially contaminated areas of the catchment. Indeed, normalized concentrations > 1 of all metals mainly at mid- (D4 and D5) and downstream (E2 and D6) sampling sites (Fig. 6.1B) suggest the existence of significant metal inputs in these locations.

Table 6.1 clearly shows that anthropogenic influence increases, to a greater (Pb and Cr) or a lesser (Mn and Ni) extent, from the bulk to the fine fraction. In the case of the bulk surface sediments, only the median EF values for Cu and Zn show moderate anthropogenic pollution. However, metal pseudo-contents in the fine surface sediments represent a moderate (Pb, Cr and Ni) or even significant (Zn and Cu) enrichment. The extreme anthropogenic pollution at E1, due primarily to Zn and Pb (Table 6.1), should be noted. This contradicts the premise that the headwater of the Ego tributary is a non-disturbed area. Vehicle emissions from the main national road and motorway (Fig. 6.1B) might be the main source of these metals in surface sediments (Saeedi et al., 2009; Adamiec et al., 2016). It is also interesting to note the strong relationship between DF and GEF for the fine fraction (Fig. 6.3A), which suggests that anthropogenic sources of metals are responsible for the anomalies throughout the catchment, especially in the Ego tributary (E1 and E2), and mid- (D5) and downstream (D6) of the main river. In the case of the bulk fraction, the absence of a linear relation between factors (Fig. 6.3A) indicates that anomalies are fundamentally due to metal contribution from nature sources.

The results of sequential extraction of metals (Fe, Mn, Zn, Cu, Ni, Pb and Cr) in the bulk and fine surface sediments are shown in Table 6.2. The concentration of all metals in the three mobile fractions (F_1, F_2 and F_3) significantly increased ($\rho < 0.05$) with decreasing grain size, except for Fe and Cr in the exchangeable and acid-extractable fraction (F_1), and for Mn, Zn, Ni and Cr in the oxidizable fraction (F_3). In contrast, the proportion of all metals in the residual fraction (F_4) significantly decreased ($\rho < 0.05$) with decreasing grain size, except for Cr. The higher percentages of the mobile fractions in the < 63 μm particle size suggest a preference of the available metals —mainly attributed to an anthropogenic input (Ramirez et al., 2005; Tiquio et al., 2017)— for smaller particles. Meanwhile, the predominance of the residual fraction in the < 2 mm grain size indicates that metals were strongly retained in the

mineral lattice and, consequently, that they were largely contributed by lithogenic sources (Kang et al., 2017).

The mean ICF (Table 6.1) of metals are ranged in the order Pb > Mn > Zn > Cu > Ni > Cr > Fe, with no variation between particle sizes. Considering that ICF reflects the risk to a water-sediment body of a pollutant, the bulk surface sediments only represented a risk (ICF > 1) from Mn and Pb, especially at D1. Conversely, all metals in the fine surface sediments were widely available in all sampling sites, mainly at E2, D3 and D6. The strong positive relationships between GCF and DF (Fig. 6.3B), and GCF and GEF (Fig. 6.3C) for the fine surface sediments indicate that anomalies found throughout the catchment were due to the most mobile metals, which were likely to come from anthropogenic sources. Conversely, the negative relationship between GCF and DF (Fig. 6.3B) for the bulk surface sediments suggests that metals in the residual fraction (F_4) and, consequently, lithological characteristics of each sub-basin were responsible for the anomalies.

In order to obviate the greater abundance of metals in the residual fraction of the bulk surface sediments than in the fine surface sediments, and to identify different metal partitioning in the entirely non-residual fraction (F_{123}) depending on particle size, the results for the mobile fractions were recalculated (unpublished data) based on their sum ($F_1' = F_1/F_{123}$; $F_2' = F_2/F_{123}$; and $F_3' = F_3/F_{123}$, respectively). Two metal behaviours were distinguished:

a) The chemical distribution of Mn and Cr did not vary between grain sizes and the results are consistent with the study by Kang et al. (2017). The greatest proportion of Mn was mainly associated with F_1' (44.9-55.4%), followed by reducible (F_2') and oxidizable (F_3') fractions (31.7-37.3% and 7.32-23.5%, respectively). Although sensitive to reducing conditions, the relative predominance of Mn in the exchangeable fraction was due to compounds of this element being solubilized in surface sediments submitted to continuous changes in their redox state (Devesa-Rey et al., 2010). The highest proportion of Cr was mainly associated with F_3' (74.3-94.3%), followed by F_2' and F_1' (4.09-24.1% and 1.65-1.71%, respectively). The widespread presence of Cr in the oxidizable fraction might be related to the fact that in oxidation state, this metal could be associated with organics or adsorbed in hydrous form onto sediments (Filipek and Owen, 1979).

Table 6.2. *Metal distribution in the bulk (< 2 mm) and the fine (< 63 µm) surface sediments for all sampling sites (N = 7). The mean and standard deviation (± SD) data are presented in µg g^{-1} for all metals, except for Fe (mg g^{-1}). The value in parentheses show percentage of elemental concentration, where the highest percentage for each metal is shown in bold. Sum: $F_1 + F_2 + F_3 + F_4$; the Total was determined by microwave assisted acid digestion; Recovery (%): (Sum/Total) x 100*

	F_1	F_2	F_3	F_4	Sum	Total	Recovery
	Exch./Carbonates	**Reducible**	**Oxidizable/Sulphides**	**Residual**			
Bulk							
Fe	0.057±0.036 (0.106)	0.798±0.315 (1.48)	0.970±0.395 (1.80)	52.1±15.7 (**96.6**)	53.9	54.2	99
Mn	175±98.2 (22.1)	117±52.8 (14.7)	83.4±39.5 (10.5)	417±205 (**52.6**)	793	795	100
Zn	56.0±40.1 (16.53)	49.0±34.4 (14.5)	58.0±23.9 (17.1)	176±33.7 (**51.9**)	339	315	108
Ni	3.99±1.57 (9.83)	3.67±2.72 (9.05)	2.91±2.45 (7.17)	30.0±12.9 (**74.0**)	40.6	42.0	97
Cu	1.00±1.08 (1.62)	0.386±0.616 (0.623)	18.8±10.0 (30.3)	41.8±15.5 (**67.5**)	62.0	55.5	112
Pb	0.181±0.141 (0.844)	1.84±1.59 (8.59)	9.20±4.15 (43.0)	10.2±9.04 (**47.6**)	21.4	22.0	97
Cr	0.243±0.359 (0.531)	0.433±0.540 (0.943)	10.3±8.20 (22.5)	34.9±15.1 (**76.1**)	45.9	43.8	105
Fine							
Fe	0.030±0.024 (0.104)	4.36±1.08 (15.1)	2.41±0.786 (8.36)	22.0±3.59 (**76.4**)	28.8	29.1	99
Mn	405±229 (**48.9**)	290±209 (35.0)	40.7±21.7 (4.91)	92.4±37.5 (11.2)	828	698	119
Zn	219±263 (32.6)	299±320 (**44.6**)	74.2±74.5 (11.1)	78.9±60.14 (11.8)	670	710	94
Ni	13.7±6.39 (23.2)	18.8±7.05 (**31.8**)	8.44±4.17 (14.3)	18.1±5.49 (30.7)	59.0	53.4	110
Cu	9.82±16.6 (9.43)	35.0±30.0.7 (33.6)	41.6±17.8 (**39.9**)	17.8±10.6 (17.1)	104	108.4	96
Pb	2.27±1.65 (4.03)	38.6±12.4 (**68.5**)	15.0±13.6 (26.6)	0.487±1.29 (0.864)	56.4	48.9	115
Cr	1.48±3.02 (1.42)	11.1±12.8 (10.6)	40.0±55.9 (38.3)	51.7±27.1 (**49.6**)	104	99.2	105

b) The chemical distribution of Fe, Pb, Cu, Ni and Zn varied between sediment fractions, with the influence of the different composition and properties of particle sizes particularly noticeable on the behaviour of metals in the water environment (Yao et al., 2016). The oxidizable fraction (F_3') was the most abundant non-lithogenous fraction (57.5-93.2%) for Cu in both grain sizes. Its high affinity to humic substances (Davutluoglu et al., 2011) favours a pronounced tendency for complexation with sediment organic matter. However, the predominance of the reducible fraction (F_2') over the exchangeable fraction (F_1') in the fine surface sediments might be related to the higher content of Fe/Mn oxides and hydroxides (F_2; Table 6.2).The prevalence of Fe in the reducible fraction of the fine surface sediments (F_2'; 64.25%) also appears to influence the partitioning of Zn, Ni and Pb in the smallest particle size. Pb bound to F_2' (71.2%) exceeds Pb bound to F_3' (25.0%) in the fine surface sediments, despite the tendency of lead to form stable organic complexes and/or to be bound to sulphides (Tüzen, 2003). Although Ni and Zn were distributed between three mobile fractions in both grain sizes, they were also mainly bound to F_2' (46.9%) in the fine surface sediments.

Regardless of chemical distribution changes in F_{123}' between the bulk and the fine surface sediments, it was observed that the reducible (F_2') and the oxidizable (F_3') were the fractions which showed significant differences between particle sizes ($\rho < 0.038$) for all metals, except for Ni. In addition, although fine sediments have greater particulate organic carbon (Strom et al., 2011), Fe/Mn oxyhydroxides appeared to be chiefly responsible for the increased adsorption of metals on fine surface sediments to the detriment of organic matter.

Finally, PCAs were performed on the metal percentages in the mobile fraction (F_{123}) to elucidate the main sources of available metals in the bulk and fine surface sediments from the Deba River catchment. The two sets of results were plotted together in Fig. 6.4 to establish similarities/dissimilarities between grain sizes. PCA produced two principal components, representing 75.3% (PCI: 46.1%; PCII 29.3%) and 71.4% (PCI: 36.2%; PCII: 35.2%) of the total variance for the bulk and the fine fraction, respectively.

Based on the variable loading and sample score, similar clustering of sampling sites could be observed for both particle sizes. However, while the mobile fraction of metals shows

a widespread spatial distribution in the bulk surface sediments (from 24.1% to 62.6% of deviation between sampling sites, Fig. S6.4A), it remains unchanged along the catchment in the fine surface sediments (from 1.3% to 18.2% of deviation, Fig. S6.4B), hindering the identification of metal sources. Thus, as with the mineralogical analysis, the < 2 mm sediment grain size is more useful for identifying different natural or anthropogenic sources of available metals in the study area, while they are masked by the higher influence of downstream transport of the fine surface sediments, as discussed in the section below.

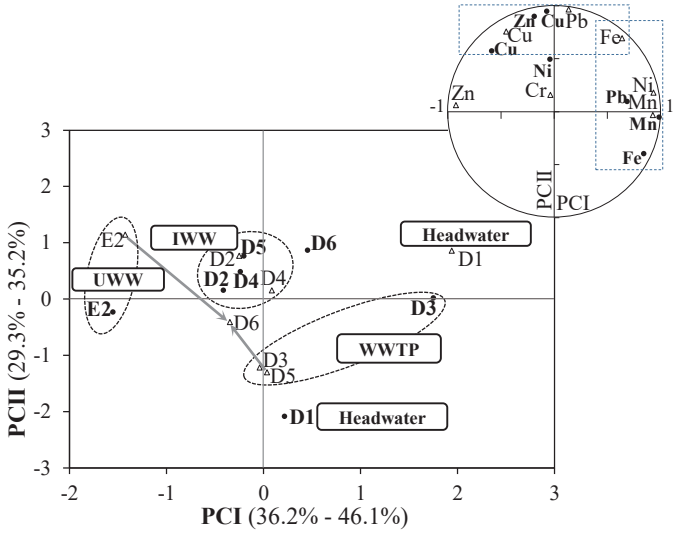

Fig. 6.4. *Principal Component Analysis applied to the mobile fraction (F_{123}) of all metals in surface sediments for all sampling sites (except for E1) and for both grain sizes (< 2 mm: triangles; < 63 µm: circles)*

Unlike the fine fraction, the bulk surface sediments from the headwater of the Deba River (D1) were characterized by having the highest available Fe, Mn, Ni and Pb percentages (8.98%, 86.9%, 58.3% and 79.6% respectively; Fig S6.4A). Their maximum percentages in the reducible fraction at D1 (Fig. S6.4A) and the absence of human activities in this area suggest that these available metals resulted primarily from natural weathering of minerals and their subsequent adsorption into the coatings formed by Fe and Mn oxides on the sandy fraction. In contrast, industrial wastewaters (IWW) were the main sources of non-lithogenous Cr in the bulk and the fine surface sediments, especially at D4 (46.6%; Fig. S6.4A and 51.4%; Fig. S6.4B, respectively). They also provided available Cu (42.6%), Zn (42.5%; mainly

at D2), and Pb and Ni (65.5% and 40.1%, respectively; mainly at D4) to the bulk surface sediments. Before connection to the Apraitz WWTP in June 2014, untreated wastewater (UWW) from Ermua appear to have contributed to the fact that the highest percentages of available Zn and Cu (63.9% and 44-0%, respectively; Fig. S6.4A) were found in the bulk surface sediments, and Cr (65.4%, Fig. S6.4B) in the fine surface sediments from the Ego tributary (E2). Although residential waste is the chief source of copper in river sediments (Zhai et al., 2003; Paramasivan et al., 2015; Yao et al., 2016), vehicles have been also reported to be one of the contributors to Zn and Cu entering the surface water system, as a result of the combustion of motor fuel, wear on brake linings, tyre wear and car washing (Legret and Pagotto, 1999; Sörme and Lagerkvist, 2002; Rule et al., 2006). Moreover, urban wastewaters are gathered from homes, commercial establishments, industries and storm-water runoff from roads, which are all also likely to be sources of metals.

Otherwise, although effluents from WWTPs are not one of the major pathways of available metals to the bulk surface sediments, Epele WWTP (D3) appears to contribute non-lithogenous Mn (63.5%), Zn (50.0%) and Ni (25.8%). In the case of the fine surface sediments, it contributes to highly mobile Fe (31.0%), Mn (94.3%), Zn (85.2%) and Ni (68.7%). Meanwhile, the Mekolalde WWTP (D5) is grouped together with IWW due to the higher available Cr, probably from D4. WWTPs treat not only urban but also industrial wastewaters, whose dissolved Zn, Ni and Mn has the lowest removal efficiency in the activated sludge process (Yamagata et al., 2010; Ong et al., 2010; da Silva Oliveira et al., 2007). Finally, while available metals in the bulk fraction at D6 came from the Ego tributary (Fe, Zn, Cu and Pb) and upstream (D5; Mn and Zn), one possible anthropogenic source of non-lithogenous Fe, Mn, Ni and Cu in the fine fraction should be noted (the Apraitz WWTP or IWW).

6.4.3. Transport of contaminants by SPM

The median discharge (Q) value obtained for the 9 sampling campaigns was 10 m^3 s^{-1}. This allows us to establish two different sets of hydrological conditions: a wet season corresponding to sampling months with Q values of over 10 m^3 s^{-1} (January, February and March 2015, and January 2016); and a dry season comprising months with Q values of below 10 m^3 s^{-1}. Fig.6.5 summarizes the SPM content in the water samples and the concentrations

of Fe, Mn, Zn, Ni, Cu, Cr and Pb in the SPM in the dry and wet season. Metal content in the SPM shows significantly higher median values during the dry season than during the wet season ($\rho < 0.05$), while the SPM concentration in water samples displayed the opposite behaviour. Indeed, a decrease in water discharge causes less erosion, and thus reduces the transport capacity of the river (Pascaud et al., 2015). However, during low discharge events, resuspended particles tend to be smaller (Palleiro et al., 2013), having high metal concentrations as we observed above for surface sediments.

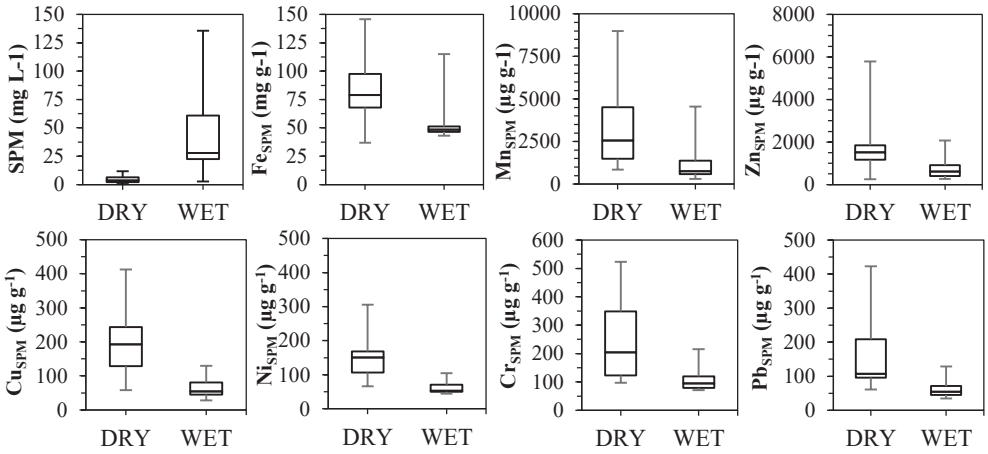

Fig. 6.5. *Box plot summarizing suspended particle matter (SPM) concentration in water samples and metal content in SPM during the dry ($Q < 10$ m^3 s^{-1}) and wet ($Q > 10$ m^3 s^{-1}) seasons. Each box shows the 25th, 50th and 75th percentiles of median values measured at each sampling site ($N = 9$ sampling campaigns)*

For a more accurate evaluation of sediment dynamics during the two different hydrological conditions, linear regressions were applied using metals as chemical connectors between SPM and surface sediments (Table 6.3). The influence of horizontal sediment transport was represented by the metal concentrations in SPM at the site just upstream (A; Table 6.3); in the case of D4 and D6, sediment transport from the tributaries (the Oñati and the Ego streams, respectively) was also taken into consideration. At the same time, the influence of vertical turbulence causing sediment resuspension was represented by metal concentrations in both the bulk (B; Table 6.3) and the fine (C; Table 6.3) surface sediments at the sampling site studied.

Table 6.3. *Derived equations for metal content (Me = Fe, Mn, Zn, Cu, Ni, Cr or Pb) in suspended particulate matter ($[Me]_{SPM}$) at each sampling site when metal concentration in suspended particulate matter from upstream site(s) and concentration in the bulk ($[Me]_{BSS}$) and the fine surface sediments ($[Me]_{FSS}$) are used as independent variables. Concentrations are expressed in μg g^{-1} for all metals, except for Fe (mg g^{-1}). The independent variable with the highest influence on the dependent variable (β absolute value) is shown in bold*

Site	Season	Equation	R^2	β values		
		A = Log $[Me]_{UPSTREAM\_SPM}$; B = Log $[Me]_{BSS}$; C = Log $[Me]_{FSS}$		A	B	C
D1	Dry	$Log\ [Me]_{SPM} = 0.66 - 0.28B_{D1} + 1.36\ C_{D1}$	0.94	*	-0.31	**1.27**
	Wet	$Log\ [Me]_{SPM} = 0.56 + 0.35B_{D1} + 0.67C_{D1}$	0.99	*	0.38	**0.62**
D2	Dry	$Log\ [Me]_{SPM} = 0.028 + 0.18A_{D1} + 0.32B_{D2} + 0.63C_{D2}$	0.99	0.16	0.32	**0.56**
	Wet	$Log\ [Me]_{SPM} = -0.032 + 0.61A_{D1} + 0.24B_{D2} + 0.066C_{D2}$	0.94	**0.65**	0.28	0.07
D3	Dry	$Log\ [Me]_{SPM} = 0.24 + 0.47A_{D2} - 0.14B_{D3} + 0.68C_{D3}$	1.0	0.43	-0.15	**0.71**
	Wet	$Log\ [Me]_{SPM} = 0.15 + 0.65A_{D2} + 0.039B_{D3} + 0.27C_{D3}$	1.0	**0.62**	0.050	0.35
D4	Dry	$Log\ [Me]_{SPM} = -0.13 + 0.61A_{D3} + 0.32A_{O1} - 0.001B_{D4} + 0.15C_{D4}$	0.99	**0.54** (D3) 0.35 (O1)	0.000	0.12
	Wet	$Log\ [Me]_{SPM} = -0.073 + 0.98A_{D3} + 0.29A_{O1} - 0.19B_{D4} - 0.042C_{D4}$	0.99	**0.92** (D3) 0.32 (O1)	-0.20	-0.044
D5	Dry	$Log\ [Me]_{SPM} = 0.92 + 1.7A_{D4} - 0.80B_{D5} - 0.36C_{D5}$	0.95	**1.9**	-0.68	-0.35
	Wet	$Log\ [Me]_{SPM} = -0.33 + 0.78A_{D4} + 0.43B_{D5} + 0.066C_{D5}$	0.99	**0.62**	0.33	0.057
D6	Dry	$Log\ [Me]_{SPM} = 0.10 + 0.42A_{D5} + 0.34A_{E2} + 0.14B_{D6} + 0.22C_{D6}$	0.99	**0.38** (D5) 0.36 (E2)	0.14	0.19
	Wet	$Log\ [Me]_{SPM} = -0.86 - 0.47A_{D5} + 1.1A_{E2} + 0.21B_{D6} + 0.86C_{D6}$	0.99	-0.40 (D5) **0.63** (E2)	0.18	0.63
E1	Dry	$Log\ [Me]_{SPM} = 0.92 + 0.59B_{E1} + 0.090C_{E1}$	0.98	*	**0.90**	0.12
	Wet	$Log\ [Me]_{SPM} = 0.47 + 0.54B_{E1} + 0.37C_{E1}$	0.92	*	**0.65**	0.39
E2	Dry	$Log\ [Me]_{SPM} = -0.30 + 0.082A_{E1} + 0.049B_{E2} + 1.1C_{E2}$	0.99	0.053	0.046	**0.93**
	Wet	$Log\ [Me]_{SPM} = 0.39 + 1.0A_{E1} - 0.11B_{E2} - 0.19C_{E2}$	0.93	**1.3**	-0.16	-0.26

As the absolute β values indicate, horizontal sediment transport was dominant during the wet season ($Q > 10$ m^3 s^{-1}). In contrast, during the dry season ($Q < 10$ m^3 s^{-1}) metal concentrations in the SPM were more importantly dependent on metal contents in resuspended surface sediments, except at D4, D5 and D6. The findings suggest that the shallow depth of the water column due to low river discharges may cause currents to approach the bottom, resulting in stronger surface sediment resuspension. However, as the discharge in the main river notably increases through streams flowing into it and the slope of the catchment abruptly decreases from D3 (Fig. 6.1C), the depth of the influence zone and the velocity of the current both rise, promoting horizontal transport over surface sediment resuspension at sites downstream of D3 and the most important tributaries (D4, D5 and D6). Indeed, the outlet of the catchment (D6) is highly influenced by sediment transport from the Ego tributary in the wet season (Table 6.3). These results concur with the earlier interpretation of the PCA (Fig. 6.4) and the study by Fdez-Ortiz de Vallejuelo and colleagues (2017), who concluded that the SPM with the highest concentrations of Zn, Pb and Cr were collected in the Altzola gauging station during flood events as a consequence of receiving waters from the Ego tributary.

Finally, it is important to stress the need to consider particle size distribution during the surface sediment resuspension event for controlling transportation of metals towards the coastal area. In effect, during the dry season, metal concentrations in the SPM originating from sediment resuspension (B and C; Table 6.3) were much more dependent on metal content in the fine surface sediments (except at D5 and E1), whereas during the wet season, they depended more on the metal content in the bulk surface sediments (except at D1, D3, D6 and E2).

6.5. Conclusions

Recognition of the susceptibility of an urban environment due to the increasing pressure from human activity or even from climate change should encourage us to gain a deeper understanding of the environmental geochemistry of metals. Since several studies have found that environmental and human health is associated with excessive exposure to metals, and sediments act as storage and transport vectors, determining the role played by sediment particle size is a valuable tool for accurately evaluating the mobilization, deposition

and dispersion of potentially toxic metals in urban ecosystems.

In this study, the use of non-destructive techniques (XRD, IR and SEM-ED spectroscopies) and invasive procedures (pseudo-total and sequential metal extraction methodologies) to analyse both bulk (< 2 mm) and fine (< 63 μm) surface sediments has allowed us to assess the potential environmental and health implications of metal geochemistry in an urban catchment. We observed that the composition and properties of each particle size influence the availability, and by extension, the toxicity of metals in surface sediments; the fine fraction mobilized not only higher metal pseudo-contents but also greater available metal percentages. In addition to the larger surface area per unit mass and the high content of clay minerals such as illite, mainly Fe and Mn oxyhydroxides have been found to encourage adsorption of metal on the smallest sediment particles, especially for Pb, Ni and Zn. In addition, we have demonstrated that discharges of IWW, UWW and even effluents from WWTPs along the Deba River catchment were the main anthropogenic sources of available metals in surface sediments. In the case of the bulk fraction, the origin of the metals was predominantly lithogenic and due to water erosion, which was identified as the dominant geomorphological force, was responsible for the presence of high available metals mainly at the headwater of the main river.

Finally, since each fraction represents different available metal loading, by evaluating sediment dynamics during different hydrological conditions, we were able to identify the season posing the highest ecological risk. While the resuspension of fine surface sediments notably induced significantly higher metal concentrations in SPM during dry season, resuspension of bulk surface sediments and, fundamentally, downstream transport of SPM became more relevant and diminished the ecological risk during the wet season. This knowledge will be crucial for sustainable development of an urban ecosystem in the context of climate change, given that future hydrological scenarios establish longer dry periods between precipitation events (IPCC, 2013), a situation in which balancing the intense expansion of human activities with the increasingly adverse natural conditions will be a very challenging task.

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6.7. Supplementary material

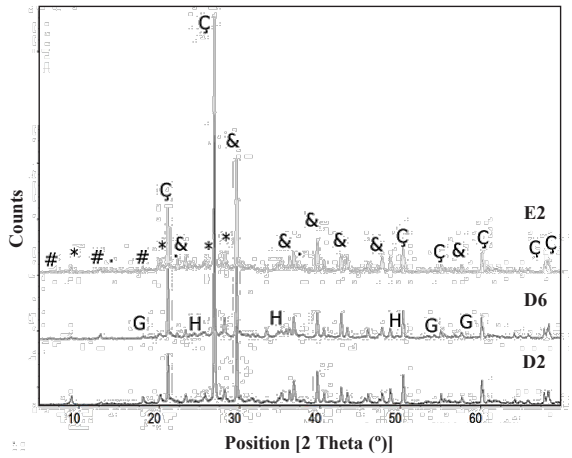


Fig. 6.S1. *XRD spectra of the bulk surface sediments from D6 and D2, and of the fine surface sediments from E2. Key: #: clinochlore; *: illite; &: calcite; ·: albite, and Ç: quartz. The main peaks of hematite (H) and goethite (G) are highlighted*

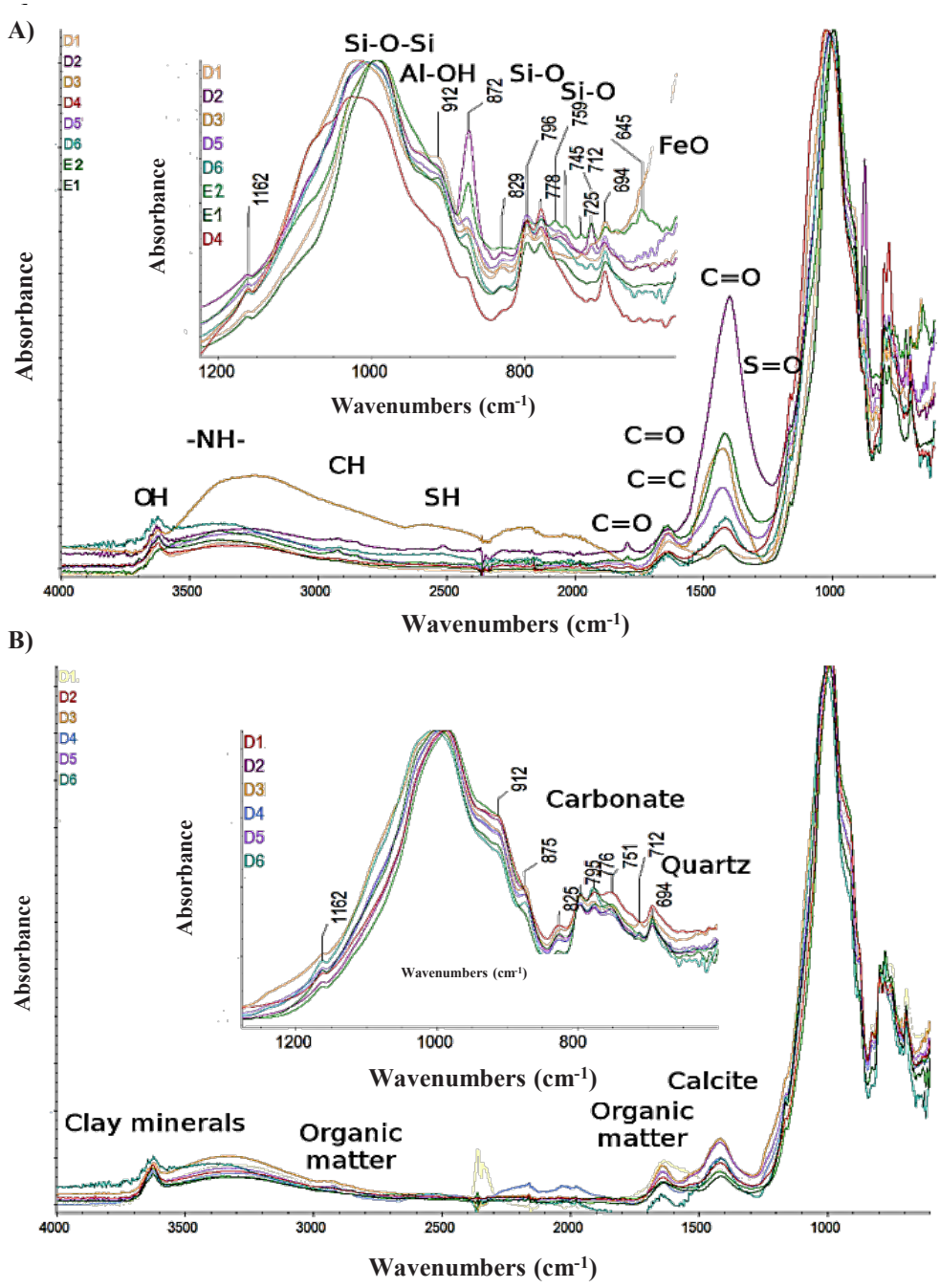


Fig. 6.S2. *MIR spectra of the bulk (A) and the fine (B) surface sediments samples from all sampling sites, except for E1 and E2 in (B)*

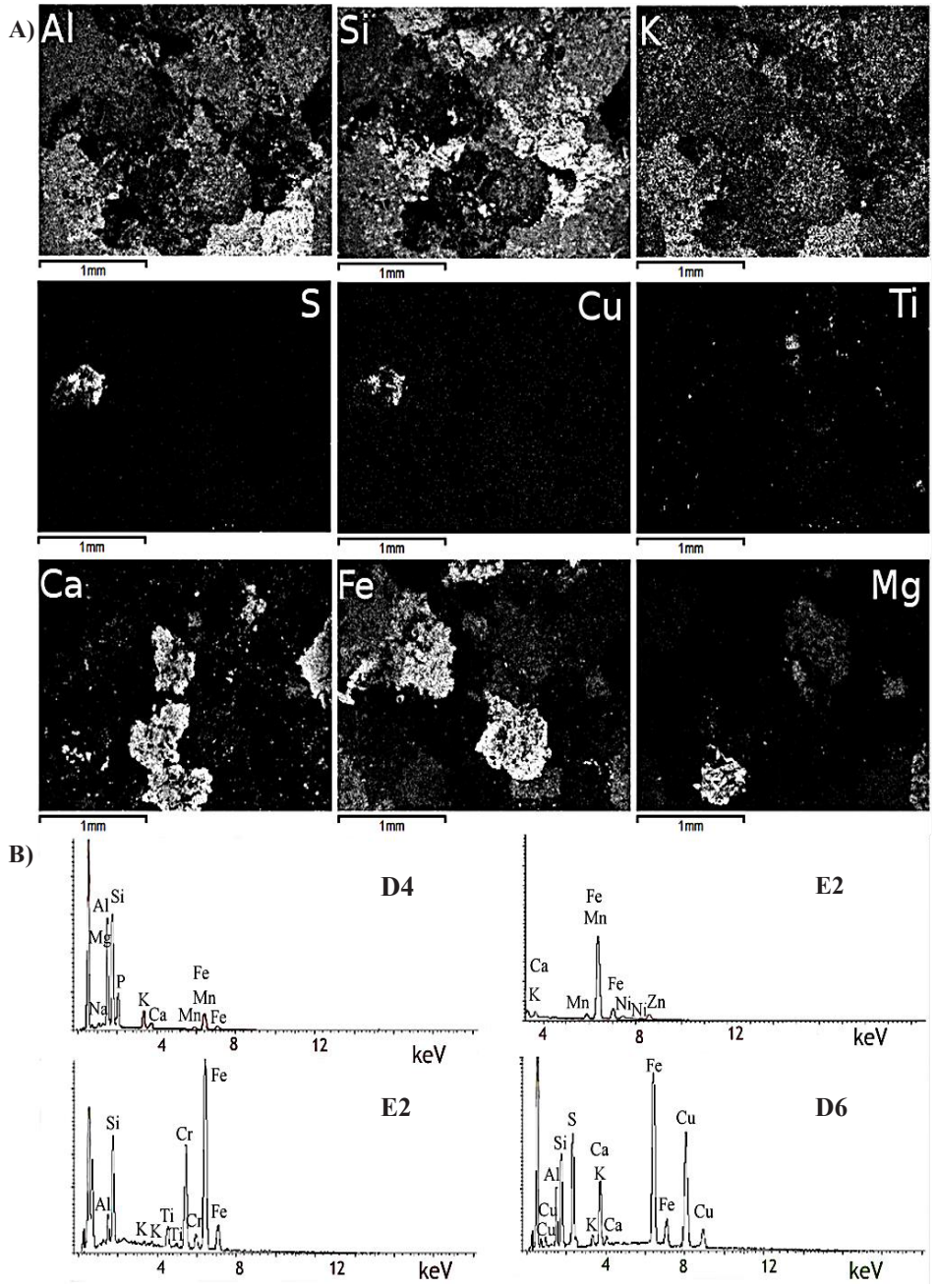


Fig. 6.S3. *As an example of the analytical technique, SEM-EDS distribution maps for aluminium, potassium, sulphur, cooper, titanium, calcium, iron, and magnesium of D6 pellet (A) and EDS spectra of D4, E2 and D6 pellets (B). For the purposes of interpretation of (A) note that the greater the proportion of each element, the brighter white it is shown in the image*

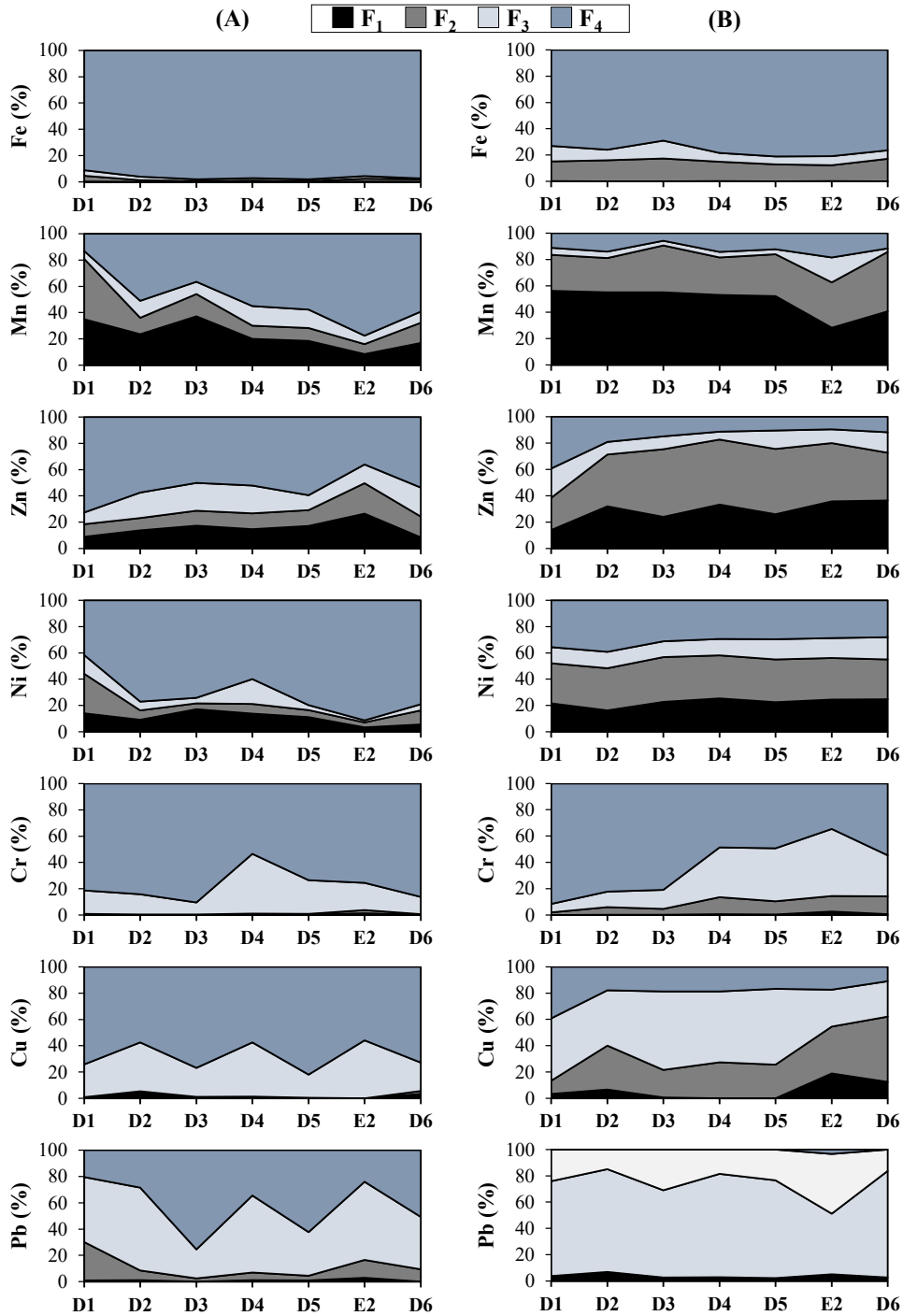


Fig. 6.S4. *Metal distribution in the four fractions considered by BCR 701 (F_1, exchangeable/carbonates; F_2, reducible; F_3, oxidizable/sulphides; F_4, residual) for the bulk (A) and the fine (B) surface sediments, and for all sites (except E1)*

Results and discussion III

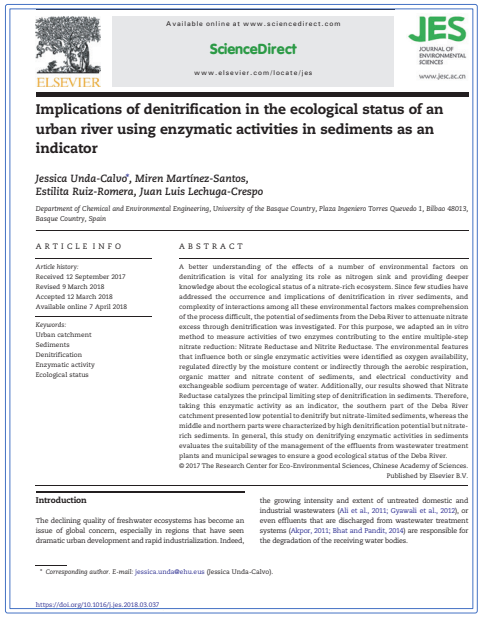
Chapter 7. Implications of denitrification in the ecological status of an urban river using enzymatic activities in sediments as an indicator

Chapter 8. Treated and untreated wastewater effluents alter river sediment bacterial communities involved in nitrogen and sulphur cycling

7

Results and discussion III

Implications of denitrification in the ecological status of an urban river using enzymatic activities in sediments as an indicator



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Implications of denitrification in the ecological status of an urban river using enzymatic activities in sediments as an indicator

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ABSTRACT

A better understanding of the effects of a number of environmental factors on denitrification is vital for analyzing its role as nitrogen sink and providing deeper knowledge about the ecological status of a nitrate-rich ecosystem. Since few studies have addressed the occurrence and implications of denitrification in river sediments, and complexity of interactions among all these environmental factors makes comprehension of the process difficult, the potential of sediments from the Deba River to attenuate nitrate excess through denitrification was investigated. For this purpose, we adapted an *in vitro* method to measure activities of two enzymes contributing to the entire multiple-step nitrate reduction: Nitrate Reductase and Nitrite Reductase. The environmental features that influence both or single enzymatic activities were identified as oxygen availability, regulated directly by the moisture content or indirectly through the aerobic respiration, organic matter and nitrate content of sediments, and electrical conductivity and exchangeable sodium percentage of water. Additionally, our results showed that Nitrate Reductase catalyzes the principal limiting step of denitrification in sediments. Therefore, taking this enzymatic activity as an indicator, the southern part of the Deba River catchment presented low potential to denitrify but nitrate-limited sediments, whereas the middle and northern parts were characterized by high denitrification potential but nitrate-rich sediments. In general, this study on denitrifying enzymatic activities in sediments evaluates the suitability of the management of the effluents from wastewater treatment plants and municipal sewages to ensure a good ecological status of the Deba River.

Introduction

The declining quality of freshwater ecosystems has become an issue of global concern, especially in regions that have seen dramatic urban development and rapid industrialization. Indeed, the growing intensity and extent of untreated domestic and industrial wastewaters (Ali et al., 2011; Gyawali et al., 2012), or even effluents that are discharged from wastewater treatment systems (Akpor, 2011; Bhat and Pandit, 2014) are responsible for the degradation of the receiving water bodies.

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7. Implications of denitrification in the ecological status of an urban river using enzymatic activities in sediments as an indicator

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7.1. Abstract

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7.2. Introduction

The declining quality of freshwater ecosystems has become an issue of global concern, especially in regions that have seen dramatic urban development and rapid industrialization. Indeed, the growing intensity and extent of untreated domestic and industrial wastewaters (Ali et al., 2011; Gyawali et al., 2012), or even effluents that are discharged from wastewater treatment systems (Akpor, 2011; Bhat and Pandit, 2014) are responsible for the degradation of the receiving water bodies. In this context, an effective water policy must take account of the vulnerability of aquatic ecosystems by the implementation of monitoring programmes for the catchment management. For a coherent and comprehensive classification of ecological status of rivers, the European Water Framework Directive (WFD, 2000/60/EC) establishes different biological, hydromorphological, chemical and physical/chemical parameters to be monitored by such programmes.

The biological indicators are exclusively relative to the aquatic flora, the benthic invertebrate fauna and the fishes living in water. However, bacteria have been recognized

as an essential food for protists and invertebrates, constituting the base of benthic food webs (Alongi, 1994) and, consequently, contributing to higher trophic level production. More relevantly, microbial communities harbored in the sediment play a vital role in improving water quality due to their involvement in the biogeochemical cycles of nitrogen (N), phosphorous (P) and sulphur (S), as well as organic matter demineralization and biochemical degradation (Paerl and Pinckney, 1996; Holmer and Storkholm, 2001; Wu et al., 2012; Zhang et al., 2013). In particular, the microbial removal of nitrogen in aquatic ecosystems is of great interest for researches and managers since nitrate excess is linked to eutrophication, especially in coastal marine waters (Burgin and Hamilton, 2007). From the perspective of river water quality, complete denitrification still remains the most desirable nitrate removal pathway because (i) it represents a permanent nitrogen sink (Arce et al., 2015) and, (ii) it does not depend on the availability of additional end-products coupled to other N transformation processes or even S cycling (Burgin and Hamilton, 2007).

Alternatively, the proposed hydromorphological, chemical and physical/chemical parameters are considered to support the biological indicators —expressed in terms of composition and abundance. Nevertheless, factors affecting denitrification drive not only the abundance and diversity, but also the activity of the denitrifying community (Wallenstein et al., 2006; Veraart et al., 2017). Moreover, denitrification rates have not to be related to the abundance and richness of the denitrifiers (Hallin et al., 2009; Graham et al., 2010; Veraart et al., 2017), suggesting that the presence of a denitrifying community does not necessarily imply that it is operating in the ecosystem.

On the other hand, since biological indicators are known to be very sensitive to any ecosystem perturbation (Chaer et al., 2009; Guo et al., 2012), denitrifying activities could act as a potential assessor of the degrading effects of anthropogenic activities on the natural biochemical process of nitrogen. In recent decades, there has been a proliferation of researches into complete denitrification in river sediments (Steingruber et al., 2001; Wall et al., 2005; Tatariw et al., 2013; Liu et al., 2013; Arce et al., 2014) and there is now a deeper understanding of the abiotic factors disturbing the biological nitrate-reducing process. However, denitrification is a facultative anaerobic process consisting of the dissimilatory reduction of NO_3^- to N_2 by four steps: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$, and it is sequentially catalyzed by Nitrate

Reductase (encoded by the gene *narG*), Nitrite Reductase (encoded by the genes *nirS/nirK*), Nitric Oxide Reductase (encoded by the gene *norB*) and Nitrous Oxide Reductase (encoded by the gene *nosZ*) enzymes (Zumft, 1997). The multiple interactions among all biotic and abiotic parameters make the process complex to understand, and investigation into single enzyme activity contributing to the entire nitrate-reduction reaction becomes crucial. For this purpose, numerous studies have examined all Reductases individually; however, these works have been primordially carried out in plants and soils.

Thus, the overall aim of this work was therefore to provide information on the activity of two Reductases (Nitrate Reductase and Nitrite Reductase) in surface sediments subject to different types of pollution (treated and untreated wastewaters discharge), in order to provide further knowledge of the effects of anthropogenic activities on denitrification, and consequently, on the ecological status of the Deba River urban catchment, using enzymatic activities as indicators. The specific objectives of this research were (i) to adapt an *in vitro* method for determining Nitrate Reductase and Nitrite Reductase activities in river surface sediments, by evaluating sample storage conditions, weight and moisture content, as well as liquid-to-solid ratio and duration of enzyme-extraction, incubation time and substrate availability, (ii) to analyze enzymatic activity rates during a rewetting period of samples, and (iii) to characterize physical/chemical properties of water and surface sediment samples and investigate their influence on enzymatic activities.

7.3. Materials and methods

7.3.1. Study area

The Deba River catchment (538 km^2) is located on the north-east coast of Spain (Gipuzkoa, Basque Country, Fig. 7.1). The Deba River (60 km) runs through the catchment towards the Bay of Biscay, receiving inflows from several tributaries (the Ego and the Oñati streams). A previous study (Martínez-Santos et al., 2015), offers an extensive description of the lithological characteristics, and main urban and industrial areas in the catchment.

Although the number of water treatment plants (WWTPs) has increased in the catchment in recent years, Borja and colleagues (2006) reported nitrogen loadings of over

9000 kg N day^{-1} km^{-2} in the water body and classified the Deba River catchment as one of the most polluted rivers in the province of Gipuzkoa. The Epele WWTP (Fig. 7.1) was put on continuous operation in May 2012; previously, organic-rich wastewaters from the towns of Arrasate and Oñati were discharged into the Deba River and Oñati stream via two sewers. However, the Basque Water Agency (URA, 2017), responsible for monitoring the chemical status of the rivers from the Basque Country, reported that the water body downstream of the Epele WWTP did not reach a "good status" for the concentration of nitrates in September, November and December 2016. On the other hand, despite being one of the largest towns in the area, the untreated sewage effluents of Ermua were previously discharged directly into the Ego stream. In June 2014, the sewer was connected to the Apraitz WWTP (Fig. 7.1) and the municipal wastewaters from Ermua are currently no longer discharged into the tributary. However, the Basque Water Agency still emphasizes the loss of natural characteristics of this stream due to infringements for ammonium, among other pollutants (URA, 2017).

7.3.2. Field methodology

In October 2015, surface sediment samples were collected from six sampling sites (D1, D2, D3, D4, D5 and D6) along the main river bank and from two sampling sites (E1 and E2) in the Ego tributary. These sampling locations were chosen in order to differentiate the influence of natural processes from the effects of anthropogenic pollution sources on enzymatic activities from headwaters to the outlet. As per USEPA (2001), surface sediment subsamples (0-5 cm depth) from multiple points within each sampling site were collected using a sterilized plastic spoon, sieved through a sterile net with a mesh of 2 mm, composited in the field and sealed in sterile polypropylene bags. Water samples were also taken in sterile polyethylene bottles at all sampling sites. Electrical conductivity (EC), pH and redox potential (Eh) were measured *in situ* using a Crison EC-Meter Basic 30+, Crison Micro pH 2000 and Hach ORP/Redox sonde MTC101 with an Ag/AgCl electrode, respectively. All water and surface sediment samples were stored and refrigerated in the dark and transported to the Chemical and Environmental Engineering laboratory (University of the Basque Country) on the same day.

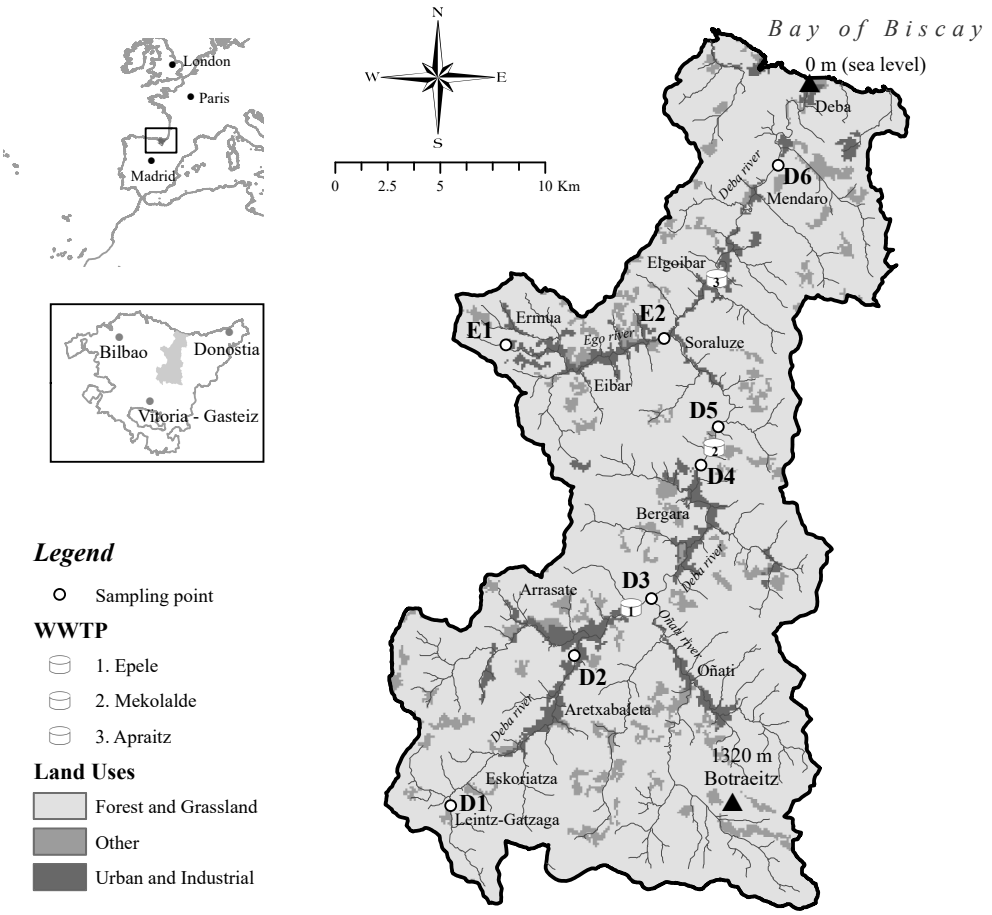

Fig. 7.1. *Location of the sampling sites, land uses, and wastewater treatment plants (WWTPs) in the Deba catchment*

7.3.3. Laboratory methodology

Surface sediments were air-dried and ground with a pestle and mortar for homogenization. Before air-drying, the moisture content of the sediment samples was determined according to APHA, 2015. One replicate of each sample was not air-dried and was directly refrigerated at 4 ºC for the purposes of performing the experiment described below.

Total carbon (TC), nitrogen (TN) and sulphur (TS) were analyzed in the surface sediments using a TruSpec CHNS determinator (Leco Corporation). Volatile solids were determined using a muffle furnace as described in Method 2540 E of the Standard Methods

(APHA, 2015). After incineration at 500ºC, the percentage weight loss was considered to be representative of total organic carbon (TOC). The inorganic nitrogen in surface sediments was determined from a KCl 1 M extraction followed by colorimetric determination of NH_4^+ and NO_3^- content (adapted from Mulvaney, 1996) using a spectrophotometer Jasco V630.

Water samples were filtered through 0.45 µm filters. One replicate of each sample was acidified to pH < 2 with HNO_3 (69%) for cations (Ca^{2+}, Mg^{2+}, Na^+ and K^+) analysis using ICP-OES (Perkin Elmer Optima 2000). Anions (Cl^- and SO_4^{2-}) were measured in the non-acidified replicate using ion chromatography (DIONEX ICS 3000).

7.3.3.1. Measurement of enzymatic activities in surface sediment samples

The following is a description of the methodology applied for measuring enzymatic activity. It is based on studies by Antolín et al. (2010) and Kakutani et al. (1981), and adapted for a sediment matrix by appropriate tests conducted with the surface sediment sample from sampling site D6. Air-dried surface sediment samples were humidified with Milli-Q until the moisture content was sufficient to ensure they were constantly moist (around 30% water). For 2 days, samples were acclimated at 13ºC to simulate autumn river conditions (river water mean temperature in October 2015).

For crude enzyme extract**,** 1 g of humidified surface sediment was immersed in 12 mL of 50 mM K-phosphate buffer (pH 7.5); 2 mM EDTA; 2 mM DTT; 1% (*W/V*) insoluble PVPP at 0ºC and 1.5% soluble casein. The mixture was continuously homogenized in a rotatory shaker for 10 min. The crude extract was centrifuged at 4300 r.p.m for 5 min and the supernatant was stored on ice. All of the operations described above were performed in a temperature range of 0-4ºC.

To perform *in vitro* Nitrate Reductase (NR) activity assay**,** 0.1 mL 50 mM K-phosphate buffer (pH 7.5); 0.1 mL NADH (1 mg mL^{-1}); 0.2 mL KNO_3 0.1 mM and 0.5 mL crude extract were made up to a final volume of 2 mL with Milli-Q. After 60 min incubation in the dark at 28ºC, the reaction was stopped by submerging test tubes in an ice bath. Assay was run in triplicate and the NR activity was expressed as the amount (µmol) of nitrites generated per gram of dry sediment and per hour.

In vitro Nitrite Reductase (NiR) activity assay was performed using initial reaction mixture contained 2 µmol of KNO_2, 0.1 µmol of methyl viologen, 20 µmol of K-phosphate buffer (pH 7.0), and 0.1 mL of the crude extract in a total volume of 0.9 mL in a test tube. After the pre-incubation for 2 min at 30ºC, the reaction was started by adding 0.1 mL of the freshly prepared dithionite solution containing 10 µmol of $NaHCO_3$ and 5 µmol of $Na_2S_2O_4$. After incubation for 90 min in the dark at 30ºC, the reaction was stopped by vigorous shaking to oxidize reduced methyl viologen and $Na_2S_2O_4$, and chilled in an ice bath. An assay was run in triplicate and NiR activity was expressed as the amount (µmol) of nitrites consumed per gram of dry sediment and per hour.

For nitrite measurement**,** 0.8 and 0.6 mL aliquots of the incubation medium from NR and NiR, respectively, were diluted to 10 mL with Milli-Q water. The amount of nitrite in the reaction mixture was analyzed by adding 1 mL of 1% (*W/V*) sulphanilamide in HCl 1.5 M and 1 mL of 0.02% (*W/V*) N-(1-naphthyl)-ethylenediamine dihydrochloride solution, and measuring the absorbance (540 nm) after 15 min by an ultraviolet spectrophotometer (Jasco V-630) (USEPA, 1979). A standard curve was prepared in the same way as the samples in the assays, but using aliquots of 0.8/0.6 mL of $NaNO_2$ standard solutions for NR and NiR, respectively.

7.3.3.2. Surface sediment sample weight, moisture conditions and storage

Sample preparation has a profound effect on the final outcome of enzyme extraction and its subsequent activity analysis. The weight-moisture tests were conducted by immersing 0.5, 1.0 and 1.5 g of air-dried (unhumidified) or wet surface sediment in enzyme-extracting dissolution. In addition, wet and humidified samples were analyzed to represent different sample storage conditions (wet or air-dried) affecting enzymatic activities with the optimal sample weight and moisture content. As both enzymatic activities were measured on aliquots from the same crude extract, NR activity was used as an indicator to select the best form of sample manipulation.

7.3.3.3. Liquid-to-solid ratio and duration of the enzymatic extraction

The step prior to the enzymatic activities assay involves the extraction of the enzymes

synthesized by the denitrifying microorganism presented in the sediment. Based on previous studies (Alche et al., 2006; Martínez-Maqueda et al., 2013), a combination of mechanical homogenization with buffers was also used since it appears to be one of the best method even for strong cell disruption in plant tissues (Van Het Hof et al., 2000). Periplasmatic and membrane-bound NR, and periplasmatic NiR take part in the denitrification pathway in bacteria (Cabello et al., 2004). It is therefore crucial to ensure an optimum contact between sediment and enzyme-extracting dissolution to break the cytoplasmic membrane and extract both enzymes. Two liquid-to-solid ratios (5:1 and 12:1) found in the literature (Kaiser and Lewis, 1984; Antolín et al., 2010) were achieved by adding 5 and 12 mL of enzyme-extracting dissolution to 1 g of humidified surface sediment. Additionally, four operating scenarios were applied with different enzyme extracting times (5, 10, 30 and 60 min) and the liquid-to-solid ratio was set at 12:1. Since there is evidence suggesting that NR is more sensitive to inactivation depending on the composition of the extracting solution than NiR (Buczek, 1984), NR activity was also used as an indicator to establish the best enzymatic extraction procedure.

7.3.3.4. Initial nitrate concentration in the NR assay and enzymatic activities kinetics

Given that surface sediments contain nitrate, the influence on Nitrate Reductase activity of adding external substrate to the incubation medium was studied. Twenty four operating scenarios were applied combining different KNO_3 dissolution concentrations (0; control, 1, 10 and 100 mM) and various incubation times (0, 15, 30, 45, 60, 75, 90, 105 and 120 min) when NR activity was measured. In addition, NiR activity assay was conducted at various incubation times (0, 15, 30, 45, 60, 75, 90, 105 and 120 min) when 0 (control) or 2 µmol of KNO_2 were added to a total volume of 0.9 mL in a test tube.

7.3.3.5. Effect of rewetting on enzymatic activity

A positive correlation has generally been found between microbial activity and water content (Iovieno and Baath, 2008). Indeed, rewetting of dry sediments has been shown to rapidly stimulate denitrification in Mediterranean temporary streams (Arce et al., 2014). However, there is little scholarship on the effect of prolonged rewetting on all Reductases that catalyze each step of the denitrification process in sediments. Therefore, air-dried surface

sediment samples were initially rewetted with Milli-Q (around 30% water) and kept moist for 5 days. The moisture content of the samples was monitored (adding Milli-Q water where necessary) and both enzymatic activities were measured daily.

7.3.4. Statistical analysis

A Shapiro-Wilk test was performed to check whether variables were normally distributed. Where necessary, data were log-transformed to ensure homogeneity of variance. The differences obtained by adjusting the parameters relative to adaptation of methodology, as well as the temporal and spatial variability of moisture content, enzymatic activities and nutrients during the rewetting period were analyzed using one-way ANOVA, taking $p < 0.05$ as significant, in accordance with Tukey's multiple range test. The physical/chemical effect of water and surface sediments on denitrifying microorganism activity was determined using Spearman correlation analysis (non-parametric test) and regression analysis between factors and NR or NiR activities. In addition, principal component analysis (PCA) was used to identify the main physical/chemical parameter controlling enzymatic activities in surface sediments from each sampling site. PCA with an eigenvalue greater than 1 was subjected to an orthogonal varimax rotation. This maximizes the variance to obtain a pattern of loadings for each factor that is as diverse as possible, thus lending itself to easier interpretation. Statistical processing of the data was performed using SPSS 22.0 software.

7.4. Results and discussion

7.4.1. Adaptation of in vitro assay of enzymatic activities for a sediment matrix

7.4.1.1. Sample preparation and enzymatic extraction

While no significant differences were found, wet surface sediment presented higher NiR activities than dry (not humidify) surface sediment, even where sample weights were different (the lowest increase was noted when 1.0 g of surface sediment sample was used in the assay). In addition, the replicate stored without previous air-drying showed a greater —though not significantly different— NR activity than the air-dried and humidified surface

sediment sample (Table 7.1). It is well-known that moisture is one of the major environmental factors controlling microbial activity in soils, which is presumably lower in sediment with low soil water content (Hicks et al., 2003; Di et al., 2014). Given this circumstance, and in order to try to preserve the sample in the best conditions, surface sediments were air-dried before storage in the expectation that enzymes would become inactive. Rewetting dry surface sediments before enzymatic analysis will result in a microbial reactivation since organic matter becomes accessible for microbial degradation and promote microorganism growth (Iovieno and Baath, 2008).

An increase in nitrite generation rates with liquid-to-solid (L:S) ratio suggests that a higher volume of enzyme extraction dissolution improves contact between the solid and aqueous phases. Also, since buffer is required to maintain the stability of enzymes in both pH and ionic-strength terms, a high quantity would appear to prevent changes in the pH of the crude extract and modification of the proteins in the sediments (Laing and Christeller, 2004). Moreover, 10 min of continuous mixture shaking seems to be enough to promote the membrane break and facilitate efflux of the enzymes into the medium (Table 7.1). Indeed, Laing and Christeller (2004) recommend that the extraction procedure be performed rapidly to minimize exposure of enzymes to potentially damaging compounds upon cell breakage.

Based on the results obtained from these different tests, and in order to reproduce faithfully the enzymatic activities occurring in the river environment, all crude enzyme extracts were obtained by immersing 1.0 g of surface sediment sample (previously humidified and acclimated for 2 days) in 12 mL of enzyme extraction dissolution and continuously shaking for 10 min.

7.4.1.2. Nitrate and nitrite reduction kinetics

The time course of nitrite accumulation during the initial 30 min of dark incubation of NR is characterized by lower NO_2^- generation when extra-nitrate is added (Fig. 7.2A). This suggests that excess substrate availability might decrease nitrite production until acclimatization and microbial demand increase. Indeed, other authors (Timpo and Neyra, 1983; Cazetta and Vasques Villela, 2004; Baloft et al., 2015) have found that, even if Nitrate Reductase activity in both leaf and stem tissues is enhanced by increases in nitrate supply, a

high NO_3^- content exceeding plant demand promotes a decrease in NR activity. Nevertheless, increasing the concentration of added KNO_3 dissolution to 100 mM results in a significant ($p < 0.05$) rise in the generation of nitrites over 60 min of incubation (Fig. 7.2A).

Table 7.1. *Nitrate Reductase (NR) activity in relation to varying parameters mentioned in the sample preparation and enzyme extraction procedure. Each value represents the mean of three replicate samples ± standard deviation. Different lowercase letters within the same parameter indicate that means are significantly different at ρ = 0.05*

Procedure	Parameter	Value		NR activity ($\mu mol\ NO_2^-$-N generated $g^{-1}\ h^{-1}$)
Sample preparation	Moisture and weight	Dry (without humidify)	0.5 g	0.530 ± 0.012[a]
			1.0 g	0.232 ± 0.015[a]
			1.5 g	0.175 ± 0.021[a]
		Wet	0.5 g	0.844 ± 0.098[a]
			1.0 g	0.245 ± 0.008[a]
			1.5 g	0.196 ± 0.013[a]
	Storage	Dry (humidify)		0.686 ± 0.021[b]
		Wet		0.746 ± 0.033[b]
Enzymes extraction	L:S ratio	5:1		0.232 ± 0.015[c]
		12:1		0.581 ± 0.016[c]
	Duration	5 min		0.563 ± 0.047[d]
		10 min		0.746 ± 0.033[d]
		30 min		0.556 ± 0.008[d]
		60 min		0.615 ± 0.034[d]

Zero-order kinetics could be used for modelling nitrite accumulation during the NR assay before maximum concentrations were achieved at 60 min, when KNO_3 is added to the incubation medium. In the case of the control replicate, surface sediment NO_3^- content was sufficient for nitrites generation but significant variations ($p < 0.05$) along the incubation time were not observed. According to linear regression, nitrate reduction coefficient was greater for KNO_3 100 mM dissolution (Fig. 7.2A). All these findings are in agreement with data from previous studies in both plants and soils (Jampeetong and Brix, 2009; Yu et al., 2012; Baloft et al., 2015), confirming that the addition of NO_3^- increases NR activity.

The NiR assay showed that the addition of external substrate (KNO_2) is crucial for enzyme activation since initial (t = 0 min) NO_2^- content in the incubation medium for the control sample was zero (Fig. 7.2B). First-order kinetics could be used for modelling nitrite accumulation during the NiR assay, just before achieving minimum concentration and adding KNO_2 (90 min). According to exponential regression, nitrite reduction coefficient was 0.013 min^{-1}. Consequently, a NR assay was conducted adding 0.2 mL of 100 mM KNO_3 to the reaction medium and incubating for 60 min, while NiR assay was run by the addition of 2 µmol of KNO_2 and 90 min of incubation.

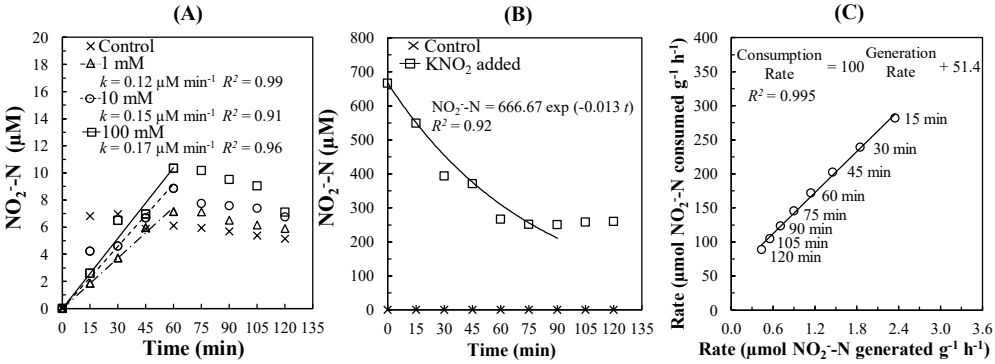

Fig. 7.2. *Time course of nitrite accumulation in the incubation medium (A) during the NR assay with varying external KNO_3 solution concentrations (zero-order models for nitrate reduction in the first hour of incubation were determined), and (B) during the NiR assay with and without KNO_2 addition (first-order model for nitrite reduction in the first 90 min of incubation was determined). The rate of nitrites consumption by NiR and the rate of nitrites generation by NR (with KNO_3 100 mM dissolution) were calculated and compared (C)*

Finally, there is evidence suggesting that NR activity, considered as a key enzyme in nitrogen metabolism, is the main limiting step in nitrate assimilation in most macroalgae and higher plants (Lea, 1997; Cazetta and Vasques Villela, 2004; Chow, 2012; Klobus et al., 2013). Therefore, the activities for both enzymes under study were calculated from nitrites accumulation data throughout the incubation period, as represented in Fig. 7.2A and B (for NR activity, results obtained when KNO_3 100 mM dissolution was added were taken into account) and compared in Fig. 7.2C. The slope of the linear adjustment (Fig. 7.2C) proves

that 100 µmol of nitrites would be consumed by NiR for each µmol of nitrite generated by NR, verifying that NR activity is also the main limiting step in nitrate reduction in sediments. This finding could be the result of (a) the abundance of *nir* gene copies as opposed to *nar* gene copies in the surface sediment; however, since activity has not necessarily to be related to the abundance and richness of the denitrifying microorganisms, gene expression studies are required for a suitable identification of those genes contributing more to nitrite-reduction than to nitrate-reduction step, and/or (b) the low conversion rates achieved by NR, which consumed nearly all the NO_3^- in the surface sediment even though around 1000 times more nitrate was added to the incubation medium (Table 7.2).

7.4.2. Enzymatic activity rates throughout the rewetting period

After humidifying (t = 0 h), the average moisture content increased to 25% ± 7% for all surface sediment samples. A slight decrease was observed to the end of the experiment (up to 39%), as shown by the fact that there were statistically significant temporal differences in moisture (Fig. 7.3A). Throughout surface sediments rewetting, NR activity rose to a peak value of 1.83 ± 0.21 µmol NO_2^--N generated g^{-1} h^{-1} at 72 h. After this time, a significant decrease was observed to the end of the experiment, evidenced by the relatively large variation (up to 55%) within the last 24 h of the rewetting period (Fig. 7.3B). On the other hand, NiR activity exhibited a non-consistent temporal trend during the test with a moderate decrease (> 27%) at 96 h of the rewetting time (Fig. 7.3C). Values decreased at the first 24 h of rewetting to a minimum of 123.1 ± 16.0 µmol NO_2^--N consumed g^{-1} h^{-1}, which increased within the next 48 h, before diminishing again to 96.6 ± 3.1 µmol NO_2^--N consumed g^{-1} h^{-1} at 96 h. NH_4^+ in the surface sediments tended to increase throughout experimental rewetting (Fig. 7.3D), and rose sharply to 2.52 ± 1.43 µmol NH_4^+-N g^{-1} at 96 h, with concentrations of 2-7 times higher than for initial conditions (t = 0 h). Finally, the NO_3^- content in surface sediments also underwent substantial changes during the test (Fig. 7.3E). All samples, except E2 and D6, exhibited a significant decrease (>42%) within the first 72 h, just before a new rise to 75% of the initial condition (0.154 ± 0.096 µmol NO_3^--N g^{-1}) at 96 h.

Median values of both enzymatic activities were not significantly different among sampling locations (Fig. 7.3B and C) due to the global effect of multiple parameters. Although

it has been argued that O_2 might be an incomplete suppressor of denitrification (Wrage et al., 2004; Vega-Jarquin et al., 2008), the denitrifying enzymes are produced in near anaerobic conditions (van Spanning et al., 2007). Therefore, when the concentration of oxygen is high, the activity of the denitrifying enzymes is expected to be inhibited (van Spanning et al., 2007; Di et al., 2014). Aside from partial oxygen pressures in the gas phase, water-filled pore space is the most important regulatory factor of soil aeration as it represents a barrier to rapid O_2 diffusion, resulting in a strong link between oxygen availability and soil water content (Smith, 1990; Weier et al., 1993; Giles et al., 2012). In this context, Nitrite Reductase activity showed a strong positive correlation with surface sediment moisture content (p = 0.541 at 0.01 level) while Nitrate Reductase activity was not correlated to this environmental factor, indicating that interactions among diverse parameters lead to different water content influence.

Contrary to expectations, enzymatic activities were not correlated to substrate availability (NH_4^+ or NO_3^- in the surface sediments) during the rewetting period. Even in saturated sediments, particle size or geometry may affect capillary action, leaving air spaces in many pores and generating some aerobic microsites, where nitrate-reducing processes could be limited (Giles et al., 2012). Thus, while *in vitro* assays measured the anaerobic nitrate and nitrite reduction rates, during the rewetting period at 13ºC both aerobic and anaerobic processes involved in nitrogen transformation might be occurring (e.g., ammonification, nitrification, denitrification, dissimilatory nitrate reduction to ammonium (DNRA), *Anammox*, etc.). NR activity, expressed as an average over two consecutive days, was compared to the variation in nitrate surface sediment content over the same period for all sampling sites. Both variables were positively correlated (p = 0.451 at 0.01 level), suggesting that anaerobic nitrate reduction catalyzed by NR enzyme took place when surface sediment samples were rewetted and acclimated at 13 ºC. However, if denitrification had been the only nitrate transformation process, the correlation would have been negative (the higher the NR activity, the greater the nitrate consumption). In addition, variation in nitrate and ammonium surface sediment contents ($\Delta\mu mol\ NO_3^-\text{-}N\ g^{-1}$ and $\Delta\mu mol\ NH_4^+\text{-}N\ g^{-1}$, respectively) over two consecutive days during the experiment were also positively correlated (p = 0.624 at 0.01 level). These results are in agreement with other authors, who reported that microbial ammonia oxidation is the first and rate-limiting step for subsequent nitrogen transformation and removal (Li et al., 2011; Zhi and Ji, 2014), since it produces substrate for Nitrate Reductase.

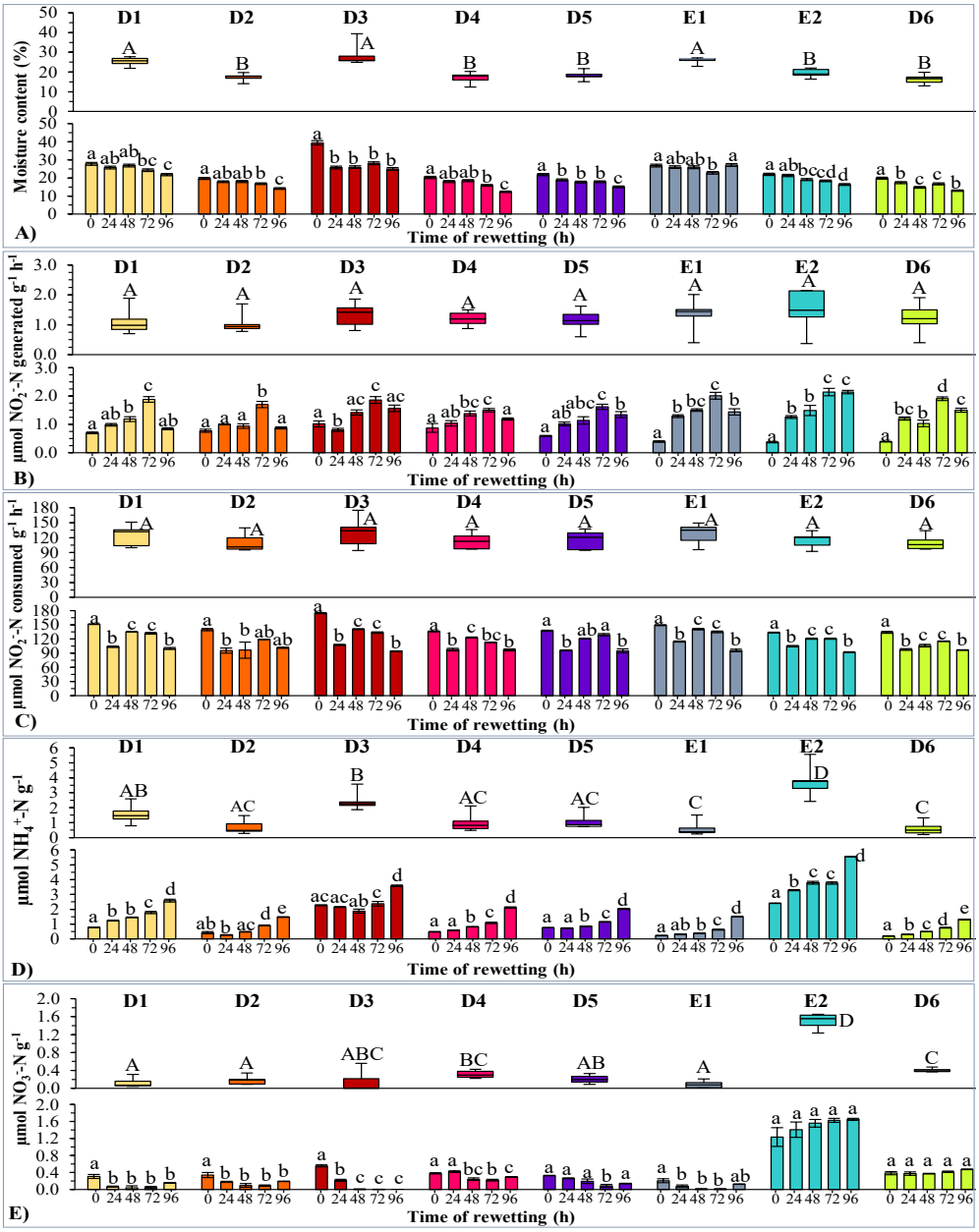

Fig. 7.3. *Moisture content (A), NR activity (B), NiR activity (C), ammonium content (D) and nitrate content (E) for all surface sediment samples are shown. Column plots represent values during the rewetting period (at 0, 24, 48, 72 and 96 h), with bars indicating the standard error (SE). Boxplots represent the distribution of values for the complete rewetting period for each surface sediment sample. Different lowercase letters within a sample indicate that means are significantly different at p=0.05 among rewetting times, while different uppercase letters indicate that medians are significantly different at p = 0.05 among sampling sites*

Finally, in the absence of a strong correlation between surface sediment moisture content and NR activity, aerobic respiration appears to play a crucial role in O_2 availability. Dissolved oxygen consumption by the oxidation of organic matter led to increasing anaerobic environmental conditions in the system, which were more favorable for denitrification (Tiedje, 1988; Giles et al., 2012; Zhi and Ji, 2014). According to previous studies (Hwang et al., 2005; Zhi and Ji, 2014), this relationship suggests an ecological and functional interaction between nitrifying and denitrifying microbial communities, inconsistent with any basic ecological premise that nitrification and denitrification are relatively independent and separate processes operating under different or contrasting conditions.

Table 7.2. *Water-surface sediment abiotic parameters and median values of NR (Nitrate Reductase) and NiR (Nitrite Reductase) activities measured in surface sediment for all sampling sites*

	Parameter	Sampling site							
		D1	D2	D3	D4	D5	D6	E1	E2
Water	EC (µS /cm)	594	1313	844	578	558	497	457	470
	pH	7.8	8.2	8.0	7.9	7.9	7.7	7.9	7.6
	Eh (mV)	185	134	143	179	196	232	230	199
	ESP (%)	44.8	44.5	31.7	22.9	24.7	23.4	7.1	12.5
Surface sediment	TC (%)	3.3	3.3	5.5	3.3	3.9	2.8	2.4	5.7
	TOC (%)	2.8	1.8	2.6	1.6	1.9	1.3	1.9	1.7
	TN (%)	0.32	0.19	0.40	0.13	0.15	0.08	0.14	0.17
	TS (%)	0.08	0.35	0.22	0.02	0.15	0.02	0.01	0.05
	TC:TN (mg TC (mg TN)$^{-1}$)	12.1	20.1	16.2	29.8	30.0	40.3	19.9	39.0
	NH_4^+ (mg kg^{-1})	9.0	4.3	20.1	7.3	6.4	3.6	3.3	36.1
	NO_3^- (mg kg^{-1})	6.0	6.0	14.6	8.4	4.4	9.4	6.2	25.8
	TOC:TS (mg TOC (mg TS)$^{-1}$)	34.4	5.3	12.0	79.3	12.4	64.0	188.6	33.0
	TOC:NO_3^- (µmol TOC (µmol NO_3^--N)$^{-1}$)	5335	3597	2106	2207	4906	1593	3578	747
	NR activity (µmol NO_2^--N generated g-1 h^{-1})	1.12	1.06	1.33	1.20	1.14	1.21	1.33	1.48
	NiR activity (µmol NO_2^--N consumed g-1 h^{-1})	125	111	130	113	116	110	127	114

7.4.3. Environmental factors affecting enzymatic activities

Electrical conductivity, pH and Eh were measured in water samples of all sampling sites (Table 7.2). In addition, exchangeable sodium percentage (ESP) was calculated as ESP (%) = $[Na^+] / [Na^+ + K^+ + Ca^{2+} + Mg^{2+}] \times 100$; where concentrations of cations are expressed

in (mg L^{-1}) (Rietz and Haynes, 2003).

The pH in the water for this sampling campaign varies between 7.7 (D6) and 8.2 (D2), indicating a weakly alkaline environment but well-buffered surface waters. Redox potential shows positive values (134-232 mV) at all sampling sites. The highest values of electrical conductivity (1313 µS cm^{-1}) and most of the major ions (Na^+, K^+, Ca^{2+}, Mg^{2+}; Cl^-, SO_4^{2-}; data not included) are found in D2. Indeed, anhydrite and gypsum intercalations with limestone and sandstones (Wealden Facies evaporates) have been reported in the upper-part of the Deba catchment by Ábalos et al. (2008) and Iribar and Ábalos (2011). Consequently, the diffuse discharge of groundwater flows from evaporitic deposits, with high content of Na^+, Cl^- and SO_4^{2-}, might influence this ionic enrichment (Martínez-Santos et al., 2015) and therefore the high values of the exchangeable sodium percentage (ESP) at D1 and D2 (44.8% and 44.5%, respectively).

Total carbon (TC) and total organic carbon (TOC), total nitrogen (TN), total sulphur (TS), total ammonium (NH_4^+) and total nitrate (NO_3^-) were also analyzed in surface sediments of all sampling sites. Additionally, TC:TN, TOC:TS and TOC:NO_3^- ratios were calculated (Table 7.2). Percentages of TN and total organic carbon (TOC) range from 0.08% to 0.40% and 1.3% to 2.8%, respectively. A significant correlation (p = 0.762 at 0.05 level) between these elements indicates that the primary source of nitrogen was the mineralization of organic matter. The spatial evolution of TOC reveals that its main sources were the natural processes and anthropogenic activities occurring throughout the catchment, such as the deposition of vegetation at D1 and E1, and WWTPs effluents at D5 and, especially at D3. On the other hand, the Ego tributary has the greatest spatial variability of nutrients; while at E1 ammonium and nitrate contents were 3.3 and 6.2 mg N kg^{-1}, respectively, E2 exhibited the highest values for these elements (36.1 mg N kg^{-1} of ammonium and 25.8 mg N kg^{-1} of nitrate). Surprisingly, the main river also shows high nutrients contents at D3 (Table 7.2). Frequently, the discharge of untreated wastewater or improperly treated effluents from WWTPs results in the deposition of large amounts of organic matter and nutrients (Naidoo and Olaniran, 2014). Even though D6 is located downstream of the Apraitz WWTP (Fig. 7.1), the decrease of TOC and the increase of nitrates compared with D5 suggest that this sampling site was more impacted by untreated urban wastewaters from the Ego tributary than by the effluents from the WWTP. At the same

time, the percentage of TS varies between 0.01% and 0.35%. Sulphate reduction below an oxygenated water column typically has TOC:TS ratios in the range of 1.5 to 5.0. A dramatic decrease in TOC:TS ratio was observed at D2, mainly caused by the high TS content (Table 7.2). This suggest that surface sediments might be deposited in periods of anoxia, although the redox potential measured in water indicates oxygenated conditions (Martínez-Santos et al., 2015).

Finally, although significant differences among sampling locations were not observed (Fig. 7.3B and C), the highest median value of Nitrate Reductase activities was computed in surface sediments at E2, followed by D3 > E1 > D6 > D4 > D5 > D1 > D2. In contrast, the greatest median value of NiR activities was observed at D3, followed by D1 > E1 > D5 > E2 > D4 > D2 > D6 (Table 7.2).

Table 7.3. *Regression analysis between median values of enzymatic activities (Nitrate Reductase (NR) and Nitrite Reductase (NiR)) and water-surface sediment abiotic characteristics*

Enzyme	Environmental factor	Regression
NR	Nitrate sediment content	$\text{NR activity} = 16.3\ NO_3^- + 1.07$
	(mg NO_3^--N/g)	$R = 0.835\ p = 0.010$
	$TOC:NO_3^-$	$\text{NR activity} = -0.159\ \text{Ln}(\text{TOC}: NO_3^-) + 2.48$
	(µmol TOC/µmol NO_3^--N)	$R = 0.752\ p = 0.031$
	Water electrical conductivity	$NR\ \text{activity} = -0.002\ EC + 2.23$
	(µS/cm)	$R = 0.810\ p = 0.051$
	Exchangeable sodium	$\text{NR activity} = -0.007\ \text{ESP} + 1.43$
	percentage (%)	$R = 0.734\ p = 0.038$
NiR	TOC	$\text{NiR activity} = 12.2\ \text{TOC} + 94.6$
	(%)	$R = 0.793\ p = 0.019$
	TC/TN	$NiR\ activity = -13.2\ \text{Ln}(\text{TC}: \text{TN}) + 160$
	(µmol total C/µmol total N)	$R = 0.721\ p = 0.044$

A number of environmental factors are known to control the rate of denitrification, including the O_2 and water content of soils, NO_3^-, carbon, pH, and temperature (Giles et al., 2012). After studying how enzymatic activities are directly or indirectly affected by O_2 and water content of sediments during the rewetting cycle, the effect of physical/chemical

characteristics of the water and surface sediment on enzymatic activities was determined by regression analysis (Table 7.3).

Some authors have highlighted the importance of substrate availability as the major environmental factor controlling microbial activity in soils (Tiedje, 1988; Iovieno and Baath, 2008; Woodward et al., 2009; Yu et al., 2012; Giles et al., 2012). As expected, a positive relationship has been found between surface sediment nitrate concentrations and Nitrate Reductase activity (NRA, Table 7.3). NRA also showed a significantly positive correlation with $N\text{-}NO_3^-$ concentration (p =0.857 at 0.01 level), indicating a greater NR activity with a greater presence of the right form of nitrogen in surface sediments. On the other hand, while nitrate surface sediment content induces Nitrate Reductase activity, TOC is only positively correlated (p = 0.857 at 0.01 level) to NiR activity, displaying a linear pattern (Table 7.3). This may be explained by the same substrate affecting the variety of Reductases differently (Giles et al., 2012). Many studies have shown that C can affect the denitrifying capacity of soils (Dodla et al., 2008; Henry et al., 2008; Yu et al., 2012; Giles et al., 2012), since its degradation provides a source of electrons for denitrifying enzymes (Richardson, 2000). However, other authors have reported that nitrate is more rapidly reduced than nitrite when there is sufficient carbon supply (Lu et al., 2014). Conversely, Henry et al (2008) found that the increasing percentage of the potential nitrate reduction rate was lower than that for potential denitrification rate in the soil microcosm amended with artificial root exudates. Observed responses indicate that organic matter content in surface sediments was sufficient for nitrate reduction while it (organic matter) was vital for the occurrence of nitrite reduction.

Carbon to nitrogen ratio appears to affect both enzymatic activities negatively (Table 7.3), in the form of $TOC:NO_3^-$ ratio to NR (p = -0.810 at 0.05 level) and as TC:TN ratio to NiR (p = -0,714 at 0.05 level). Zhi and Ji (2014) evaluated wastewater treatment performance in a wetland constructed by tidal flow under different C:N ratios (calculated as a quotient of chemical oxygen demand over ammonium-nitrogen in water), and concluded that a value greater than six was required to achieve complete denitrification. In our study, all surface sediment samples had high C:N ratios favored by nitrate-limited contents (Table 7.2). However, Burgin and Hamilton (2007) hypothesized different dissimilatory pathways of nitrate removal, and suggested that fermentative dissimilatory nitrate reduction to ammonium (DNRA) may be

favored in nitrate-limited and labile carbon-rich environments over respiratory denitrification.

Finally, NR activity decreased with a rise of water EC ($R = 0.824$; $p = 0.044$ taking into account all sampling sites, except D2 and D3) and ESP (Table 7.3), illustrating the negative effect of these parameters on the denitrifying microorganisms (Wong and Dalal, 2008). With the increased EC, denitrifying bacteria growth and respiratory activity increased, lowering the efficiency of carbon use (Yu et al., 2012). In addition, under sodic conditions, toxicities of Na^+ and other accompanying ions (e.g. Cl^- and HCO_3^-) along with water with a very high pH (around 8.0) also inhibit microbial growth (Zahran, 1997; Yu et al., 2012).

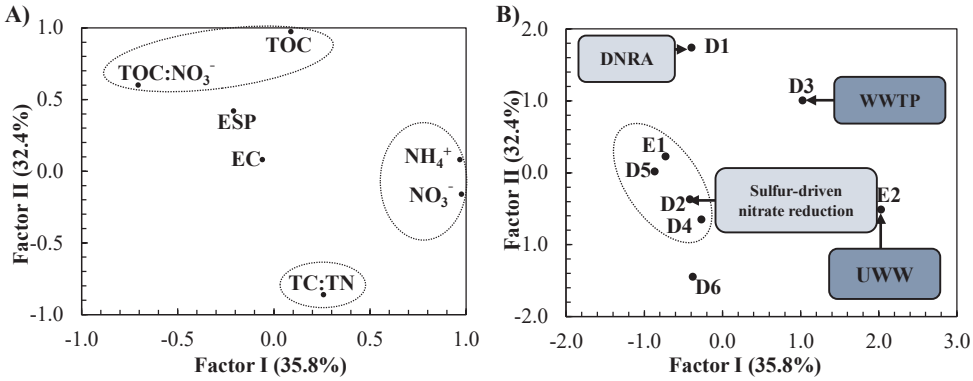

Fig. 7.4. *Loadings plot (A) and scores plot (B) of the factors to enzymatic activities in surface sediments for all sampling sites*

Since interactions among all environmental factors were considered responsible of the absence of significant spatial differences, PCA was used to identify the main factor controlling enzymatic activities at each sampling site. PCA produced two principal components, which together accounted for 68.2% of the total variance (35.8% for the Factor I and 32.4% for the Factor II). Fig. 7.4A summarizes the loadings of the factors; particularly noteworthy is the absolute value of the loadings more than 0.5 of the total variance. Factor I had strong loadings on NO_3^- and NH_4^+, while Factor II showed strong positive loadings on TOC, TOC:NO_3^-, and negative loadings on TC:TN. According to the influence of environmental parameters on enzymatic activities (Table 7.3), Factor I shows that substrate availability was a key factor for Nitrate Reductase activity. Additionally, Factor II shows that organic matter was efficiently

utilized by Nitrite Reductase, whereas TC:TN or TOC:NO_3^- ratios had a strong repression effect on both enzymatic activities.

In a plot of the scores for the factors (Fig. 7.4B), surface sediment samples were clustered as expected. E2 and D3 were the sampling sites with the highest mean NR activities due to the high ammonium and nitrate loadings from the Epele WWTP and untreated wastewaters (UWW) discharge in the Ego stream, while the high TOC:NO_3^- ratio at D1 had a negative impact on this enzymatic activity favoring the fermentative dissimilatory nitrate reduction to ammonium (DNRA). In contrast, the sampling sites with the highest mean NiR activities (D3 and D1) can be seen to be distributed towards the positive side of Factor II induced by their high organic matter content.

It was noted that sampling locations inside the confidence ellipse (Fig. 7.4B) were influenced by more than one factor and, consequently, the effects of natural processes or anthropogenic activities on enzymatic activities could not be distinguished. For example, even though an increase in TOC was observed (Table 7.2), sampling sites up- and downstream of the Mekolalde WWTP (D4 and D5, respectively) are grouped together and they are not primarily influenced by organic matter loadings (Fig. 7.4B**)**, suggesting that mineral parameters (ESP or EC) modified the effects of treated wastewaters discharge on NR and NiR activities. On the other hand, D2 had the lowest enzymatic activities even though it is not clearly associated to a main factor. Saline springs, which provide indirect evidence of the occurrence of evaporites, are common in this area and have a high Na^+, Cl^- and SO_4^{2-} concentration with an important presence of dissolved H_2S (Iribar and Ábalos, 2011). Moreover, the high sulphur content and the low TOC:TS ratio at D2 (Table 7.2) suggest that these surface sediments might have been deposited under periods of anoxia, favoring the biological sulphate reduction (Appelo and Postma, 2005) and the subsequent formation of metal sulphides (Fu and Wang, 2011; Martínez-Santos et al., 2015). In recent years, the existence of reduction of nitrate coupled with oxidation of reduced sulphur forms, including free sulphide (H_2S and S^{2-}) and elemental sulphur (S) has been documented in marine and freshwater ecosystems (Brettar and Rheinheimer, 1991; Brunet and Garcia-Gil, 1996; Burgin and Hamilton, 2007). It may therefore be concluded that the inhibition by sulphide and the high EC and ESP values observed for this site (Table 7.2) could explain the low enzymatic activities measured at D2.

Finally, the response-time of the microbial communities harbored in the surface sediments for reducing nitrates, which has been previously considered the main limiting step of denitrification, were calculated from the nitrate content and the NR activity (Table 7.2). Considering an adequate optimization of the *in vitro* method for determining the enzymatic activities, the response-time represents a "theoretical minimum response-time" of the microbial communities. Despite the high NR activity, E2 and D3 presented the highest values (75 and 47 min, respectively) compared to the rest of sampling sites (17, 20, 23, 24, 30 and 33, for D5, E1, D1, D2, D4 and D6, respectively), indicating that the denitrifying activity might not be sufficient to support the high nutrient loadings from Ermua municipality and Epele WWTP, and to ensure the ecological health of the surrounding environment. In addition, although headwaters of the Ego tributary (E1) presented approximately 50% more NO_3^- than surface sediments from downstream of the Mekolalde WWTP (D5), they both showed the lowest response-time values. Therefore, the response-time highlights the importance of considering both the cumulative effects of the pollutants concerned (nitrates) and the purifying capacity of the recipient natural environment (denitrifying activity), as a more reliable reflect of the ecological status of an ecosystem.

7.5. Conclusions

The ecological effect of nitrate excess in rivers is a worldwide problem. In this context, denitrifying microbial communities, which are involved in the biological nitrate removal, determine the capacity of aquatic ecosystems to ensure a good ecological status. However, the European Water Framework Directive does not include the analysis of denitrifying activities in the monitoring programmes for the catchment management, as a more accurate indicator of the vulnerability of rivers against any ecosystem perturbation.

Several authors have evaluated the influence of numerous biotic and abiotic factors on denitrification in sediments. Nevertheless, since it comprises the reduction of NO_3^- to N_2 by four steps, the analysis of the effects of various physical/chemical parameters (moisture, organic matter and nitrate content of surface sediment, as well as electrical conductivity and exchangeable sodium percentage of water) on the activities of the enzymes —Nitrate Reductase and Nitrite Reductase— which catalyzed the first two stages of denitrification

(NO_3^- → NO_2^- and NO_2^- → NO, respectively) has been proposed. Thus, this work intends to contribute to a better comprehension of denitrification in sediments through deeper knowledge of each enzymatic activity contributing to the complete process. We also highlight the necessity of further analyzing and understanding of alternative microbially mediated nitrate removal pathways. In fact, natural processes like fermentative dissimilatory nitrate reduction to ammonium (DNRA) or sulphur-driven nitrate reduction seem to predominate over denitrification in the headwater of the main river.

Finally, this study suggests a reliable evaluation of the ecological status of a catchment based on considering both the accumulation of pollutants in sediments and the purifying capacity of the recipient natural environment. Nitrate Reductase activity, considered the main limiting step of denitrification, was almost always higher in surface sediments subject to pollution by treated or untreated wastewaters than in non-impacted ones. Specifically, the discharge of untreated urban wastewaters into the Ego stream until June 2014 and effluents from the Epele WWTP were largely responsible for inducing the greatest Nitrate Reductase activities. However, the highest nitrate contents in those same surface sediments indicated that denitrification might not be sufficient to support the high nutrient loadings and to ensure the ecological health of the surrounding environment.

7.6. References

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8

Results and discussion III

Treated and untreated wastewater effluents alter river sediment bacterial communities involved in nitrogen and sulphur cycling

8. Treated and untreated wastewater effluents alter river sediment bacterial communities involved in nitrogen and sulphur cycling

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8.1. Abstract

Studying the dynamics of nitrogen and sulphur cycling bacteria in river surface sediments is essential to better understand their contribution to global biogeochemical cycles. Evaporitic rocks settled at the headwater of the Deba River catchment (northern Spain) lead to high values of sulphate concentration in its waters. Besides, the discharge of effluents from untreated and treated residual (urban and industrial) wastewaters increases the concentration

of metals, nutrients and organic compounds in its mid- and low-water courses. The aim of this study was to assess the impact of anthropogenic contamination from untreated and treated residual and industrial wastewaters on the structure and function of bacterial communities present in surface sediments of the Deba River catchment. The application of a quantitative functional approach (qPCR) based on denitrification genes (*nir*: *nirS* + *nirK;* and *nosZ*), together with a 16S rRNA gene metabarcoding structural analysis, revealed (i) the high relevance of the sulphur cycle at headwater surface sediments (as reflected by the abundance of members of the *Syntrophobacterales* order, and the *Sulfuricurvum* and *Thiobacillus* genera) and (ii) the predominance of sulphide-driven autotrophic denitrification over heterotrophic denitrification. Incomplete heterotrophic denitrification appeared to be predominant in surface sediments strongly impacted by treated and untreated effluents, as reflected by the lower values of the *nosZ:nir* ratio, thus favouring N_2O emissions. Understanding nitrogen and sulphur cycling pathways has profound implications for the management of river ecosystems, since this knowledge can help us determine whether a specific river is acting or not as a source of greenhouse gases (i.e., N_2O).

8.2. Introduction

Uncontrolled urbanization and industrialization in urban catchments have led to increasing contamination and environmental deterioration of river waters and sediments. In most urban catchments, the discharge of effluents from residual (urban and industrial) wastewater treatment plants (WWTP), as well as untreated (UWW) and industrial (IWW) wastewaters, presents a major challenge for the maintenance of water and sediment quality (Buzier et al., 2011; Stalter et al., 2013). Although wastewater treatment in Europe has considerably improved during the last decades (European Environment Agency, 2017), it must be remembered that effluents from WWTPs still contain nutrients, organic and inorganic contaminants, as well as large quantities of microorganisms (Shon et al., 2006; Bundschuh, 2014; Fauvel et al., 2016). Macronutrients and micronutrients, including certain metals, are both essential for the survival of living organisms in aquatic systems, but an excess of nutrients and metals can cause eutrophication (Hilton et al., 2006) and ecotoxicity (Oves et al., 2016) problems, respectively. In addition, the adverse impact of urban and industrial effluents on microbial communities can have negative consequences for aquatic ecosystems (Wakelin

et al., 2008; Chonova et al., 2016) and, in particular, for global biogeochemical cycling (Grob et al., 2013).

The release of nitrogen-rich wastewater into rivers is expected to both (i) alter nitrogen cycling pathways and (ii) affect the composition, diversity, spatial-distribution and functioning of water and sediment microbial communities. Although alternative nitrate removal pathways, such as anaerobic ammonium oxidation (Anammox), autotrophic denitrification via sulphur or iron oxidation, and dissimilatory nitrate reduction to ammonium (DNRA), are often present in aquatic systems (Burgin and Hamilton, 2007; Giles et al., 2012), incomplete heterotrophic denitrification is probably the main biological source of N_2O (a well-known potent greenhouse gas) emissions. Heterotrophic denitrification involves the reduction of NO_3^- to N_2 by four steps: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. Nonetheless, not all denitrifiers harbour the complete set of denitrifying genes to carry out the whole transformation from NO_3^- to N_2. Among all denitrifying genes, nitrite reductase genes (*nirS* and *nirK*), responsible for the reduction of NO_2^- to NO, are considered to be universal to all denitrifiers (Desnues et al., 2007; Azziz et al., 2017). Graf et al. (2014) found both genes within the same genome but this possibility appears to be an exception. The last step of heterotrophic denitrification is driven by the nitrous oxide reductase (*nosZ*) gene, responsible for the conversion from N_2O to N_2. However, many denitrifying bacteria lack this gene and, in consequence, N_2O becomes the end-product of such incomplete heterotrophic denitrification (Jones et al., 2008; Ligi et al., 2014). This makes *nosZ* an important target gene in studies of denitrifying bacteria (Philippot et al., 2009; Philippot et al., 2011; Sanford et al., 2012).

Furthermore, the sulphur cycle is especially important in river waters that have high SO_4^{2-} concentrations coming from the oxidation of H_2S from sulphur springs (Szynkiewicz et al., 2012). Reservoirs of sulphur in freshwater environments are dominated by (i) dissolved sulphate in water and (ii) sulphate and sulphide minerals in sediments (Wynn et al., 2010; Iribar and Ábalos, 2011). Sulphur cycling bacteria can use oxidized or reduced forms of sulphur, but not all sulphur cycling bacteria can use both. Anaerobic conditions, together with a supply of sulphate, can lead to sulphide production from the activity of sulphate-reducing bacteria, most of them belonging to the *Deltaproteobacteria* group (Kubo et al., 2014; Zheng et al., 2017). The sulphide produced is then available to sulphur-oxidizing bacteria

(e.g., *Betaproteobacteria*, *Epsilonproteobacteria*), thereby completing the sulphur cycle. Moreover, autotrophic denitrification can be carried out through the ability of some bacteria to couple the reduction of nitrate to the oxidation of sulphur (Burgin and Hamilton, 2007). The biogeochemical importance of autotrophic denitrification in freshwater ecosystems has previously been recognised (Haaijer et al., 2007; Burgin and Hamilton, 2008), but it is still much more poorly documented than in marine ecosystems.

Due to their key role in biogeochemical cycling and their sensitivity and quick response to changing environmental conditions, microorganisms are particularly suitable for the monitoring of river and sediment quality. In order to study the dynamics of bacterial communities involved in nitrogen and sulphur cycling in river surface sediments, as well as their responses to anthropogenic disturbances, we used a combination of two established molecular techniques: qPCR and 16S rRNA gene metabarcoding for functional (denitrification genes) and structural information, respectively.

Previous studies showed that surface sediments of the Deba River catchment (northern Spain) can act as storage and transport vectors of nutrients, metals and other harmful contaminants (Martínez-Santos et al., 2015), representing a potential risk for ecosystem and human health (Unda-Calvo et al., 2017). The main objectives of this study were to: (i) assess the impact of anthropogenic contamination from residual and industrial effluents on the structure (16S rRNA gene) and function (denitrification genes) of bacterial communities present in surface sediments of the Deba River catchment; (ii) improve our understanding of the ecological role of sulphur and nitrogen cycling bacteria in those surface sediments; and (iii) examine the suitability of the combination of functional qPCR and structural (16S rRNA gene) metabarcoding to reveal possible alterations of the nitrogen and sulphur cycles in river surface sediments. We hypothesized that residual and industrial effluents will alter the structure and functioning of river sediment bacterial communities involved in nitrogen and sulphur cycling.

8.3. Materials and methods

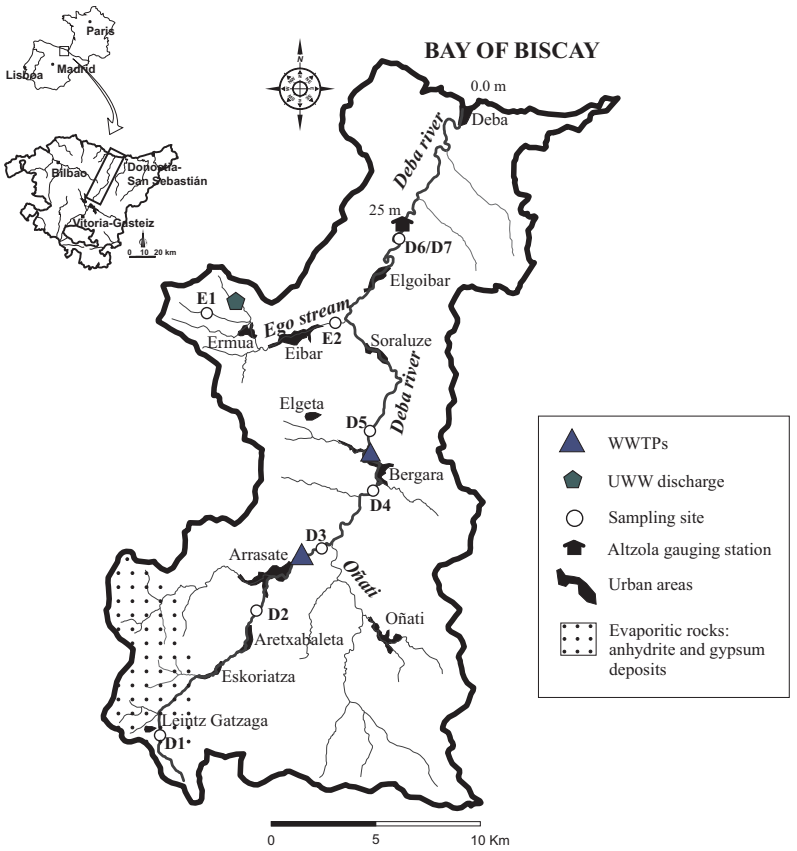
8.3.1. Site description and sampling

The Deba River catchment (538 km^2) is geologically located over evaporitic rocks (anhydrite and gypsum deposits) interlayered with sedimentary rocks, sandstones and mudstones. Saline springs with high Na^+, Cl^-, SO_4^{2-} and dissolved H_2S are common in this area (Iribar and Ábalos, 2011). Moreover, metals, nutrients and/or organic-rich compounds from residual and untreated effluents discharge into the Deba River catchment with adverse consequences for water and sediment quality. Many industries contribute to increase the concentration of metals in midstream water and sediments of the Deba River (Martínez-Santos et al., 2015). In this area, wastewater treatment plants, which collect urban and industrial effluents (residual), use primary (mechanical) and secondary (biological) treatments to remove suspended solids and decompose most of the organic matter. Tertiary (chemical) treatment is used to remove phosphorous by precipitation with Cl_3Fe (only from May 1st to October 15th). Treated effluents are neither chlorinated nor filtered before being discharged into the river.

In October 2015 (a low-flow period, i.e. 1.67 m^{-3} s^{-1}), samples of the water column and surface sediments were taken from eight different sites along the Deba River catchment: from the headwater to immediately downstream the Altzola gauging station (D1-D6/D7), and from the Ego tributary (E1 and E2) (Fig. 8.1). These eight sampling sites were chosen in an attempt to study the influence of different types of effluents (residual and industrial) on river water and sediment chemical quality, as well as on the bacterial community structure and function of surface sediments. Two types of surface sediments were found at the gauging station site: stream bed (D6, brownish-black colour) and stream bank (D7, greyish colour) sediment. Both types of sediments were independently processed and analysed for biological parameters. All the other sediment samples corresponded to bed sediments, as bank sediments were not detected in the other sites (D1-D5, E1-E2).

Sediment sub-samples (0-5 cm depth) from 5 points within each sampling site were randomly collected using a sterilized plastic spoon. Samples were then sieved through a sterile net with a mesh of 2 mm, according to USEPA (2001). Sediment samples were stored in two sterile polypropylene containers for chemical and biological analysis, respectively.

Water samples were collected in polyethylene bottles at all sampling points. Measurements of water pH were carried out *in situ* with a Crison Micro pH 2000. All water and sediment samples were refrigerated in the dark, and transported immediately to the laboratory.



Site	River/stream	Location	Contamination source
D1	Deba	Headwater	Non-impacted
D2	Deba	Mid-water course	Industrial
D3	Deba	Mid-water course	WWTP+industrial
D4	Deba	Mid-water course	WWTP+industrial
D5	Deba	Mid-water course	WWTP+industrial
D6/D7	Deba	Low-water course	WWTP+industrial
E1	Ego	Headwater	Non-impacted
E2	Ego	Low-water course	UWW+industrial

Fig. 8.1. *Location of wastewtater treatment plants (WWTPs), untreated wastewater (UWW) discharge, sampling sites, and Altzola gauging station in the Deba River catchment. The dotted area corresponds to the presence of avporitic rocks (anydrite and gypsum deposits). Table summarizes the most important information of the sampling sites: location in the catchment and the impact of different contamination sources in each site (according to the results obtained in the clusters of Fig. 8.2)*

8.3.2. Analysis of environmental parameters

Once in the laboratory, surface sediments were air-dried and ground with a pestle and mortar for homogenization. Their moisture content was determined according to APHA (2015).

Chemical analyses (3 technical replicates) were performed in the dried sediment bulk fraction (< 2 mm). Total carbon (TC), nitrogen (TN) and sulphur (TS) were analysed using a TruSpec CHNS Elemental Determinator (Leco Corporation). Volatile solids were determined using a muffle furnace as described in Method 2540 E of APHA Standard Methods (APHA, 2015). After incineration at 500°C, the percentage of weight loss was considered to be representative of total organic carbon (TOC). Total inorganic carbon (TIC) was calculated by the difference between TC and TOC. TOC:TIC and TOC:TS ratios were calculated. Inorganic nitrogen (NH_4^+ and NO_3^-) was determined after 1 M KCl extraction (adapted from Mulvaney, 1996) using a Jasco V630 Spectrophotometer.

Pseudo-total metal concentrations in dried sediment bulk fractions were also determined. An ETHOS One Microwave Digestion System (Milestone) was used to digest, with concentrated HNO_3:$HClO_4$ (3:1.5), three technical replicates from each sediment sample (0.5 g) in Teflon vessels (USEPA, 2007). Metal (Cu, Cr, Ni, Pb and Zn) concentrations were determined by ICP-OES (Perkin Elmer Optima 2000). To estimate the potential for metals to cause harmful effects in exposed people to sediments, hazard quotient (HQ) values were calculated as shown in the following equation (Eq. 8.1):

$$HQ = \frac{CDI\ x\ B/100}{RfD} \quad (8.1)$$

Chemical daily intake (CDI) represents the possible entry of metals (mg kg^{-1} day^{-1}) into the human body, B is the percentage of bioaccessible (gastric + intestinal) metal pseudo-content in surface sediments and RfD is the estimated amount of the daily oral exposure for the populations. All the analysis and calculations were made following the procedure of Unda-Calvo et al., (2017). If the sum of HQ corresponding to the gastric and intestinal bioaccessibilities for at least one of the metals (Cu, Cr, Ni, Pb and Zn) considered

at each site exceeds 1, the surface sediment may be a concern for potential harmful effects.

Water samples were filtered through 0.45 µm Milipore nitrocellulose filter. Dissolved and particulate organic carbon (DOC and POC) were analysed using a Total Organic Carbon Analyzer (TOC-L Shimadzu). Anions (NO_3^-, SO_4^{2-} and Cl^-) and cations (Ca^{2+}, Na^+, K^+ and Mg^{2+}) were measured using ion chromatography (DIONEX ICS 3000) and ICP-OES, respectively. Concentrations of PO_4^{3-}-P and NO_2^--N were determined by the ascorbic acid method (4500-P E) (APHA, 2015) and by the N-(1-naphthyl) ethylenediamine dihydrochloride method (354.1) (USEPA, 1979), respectively. The modified Berthelot reaction was used for NH_4^+-N determination in water samples.

8.3.3. DNA extraction and metabarcoding

Sediment samples for DNA analysis were stored fresh at -20°C. DNA was extracted from sediment samples (0.25 g of dry weight (DW) sediment) using Power Soil™ DNA Isolation Kit. Prior to DNA extraction, sediment samples were washed twice in 120 mM K_2HPO_4 (pH 8.0) to wash away extracellular DNA. The, the sample is centrifuged to the high speed (13,000 g), so that the supernatant (extracellular DNA dissolved in K_2HPO_4 solution) is removed and the intact cells still remain in the sediment sample (Kowalchuk et al., 2003).

Metabarcoding (amplicon) library preparations were carried out as described in Lanzén et al. (2016). The small subunit ribosomal RNA gene (16 rRNA) was amplified from prokaryotes using primers 519F and 806R targeting the V4 hypervariable region (see Table 8.S1). Adapter-linked primer pairs were used during a first PCR, followed by cleaning and a second PCR with adapters linked to a sample-specific barcodes. Pair-ended sequencing was carried out using an Illumina MiSeq with the V2 kit at Tecnalia Corporation (Miñano, Spain). Sequence data has been deposited to the European Nucleotide Archive with study accession PRJEB24857.

8.3.4. Real-time quantitative PCR analysis

Real-time qPCR (qPCR) was carried out for measurements of total bacteria (16S rRNA gene), *nirK*, *nirS* and *nosZ* gene copy abundance as described in Epelde et al. (2014), and

corrected with the respective efficiencies (16S rRNA, 98.82%; *nirK*, 96.82%; *nirS*, 92.17%; and *nosZ*, 95.02%). The limit of detection was stated as the highest quantitatively determined cycle threshold values (Ct: 16S rRNA, 29.65; *nirK*, 31.48; *nirS*, 33.24; *nosZ*, 28.60) and checked with the Melt curve. Known template standards were made from whole genomes extracted from pure bacterial isolates as described in Yergeau et al. (2007): *Escherichia coli* was used for 16S rRNA gene, *nirK* and *nirS* genes, while *Pseudomonas mendocina B* was used for the *nosZ* gene. The primers used are shown in Table 8.S1.

Each 25 µl reaction mixture contained 2.5 µl of template DNA, 12.5 µl of SYBER PremixExTaq (Takara), 2.5 µl of each primer at a concentration of 10 µM, 1.25 µl bovine serum albumin (BSA; 40 mg ml^{-1}) and 0.5 µl of ROX dye. Each sample was measured in triplicate. qPCR conditions were as follows: For total bacteria (16S rRNA gene); 95ºC for 15 min; 94ºC for 30 s; 52ºC for 30 s; 72ºC for 1 min (40 cycles); 95ºC for 15 s; 60ºC for 1min; 95ºC for 30 s for the melt curve with a final extension of 60ºC for 15 s. For *nirK*, *nirS* and *nosZ* genes: 95ºC for 15 s; 60ºC for 1min; 95ºC for 30 s for the melt curve with a final extension of 60ºC for 15 s.

8.3.5. Sequence data analysis and taxonomic classification

Sequence read-pairs were quality-filtered and overlapped using vsearch with default parameters except allowing "staggered" read pairs (Rognes et al., 2016). Paired reads were trimmed from both ends to remove N5 and primer sequences, using cutadapt (Martín, 2011), discarding any sequences not containing the full primer within one mismatch. Finally, using vsearch, sequences were truncated to 253 nt, while shorter sequences or those with low quality (fastq_maxee = 0.5) were removed. All quality-filtered overlapped sequences were clustered into OTUs using Swarm v2 (Mahé et al., 2015). Swarm OTUs were subjected to *de novo* and reference based chimera filtering (with the rdp_gold reference database), using vsearch (UCHIME algorithm). Remaining chimera-filtered Swarm OTUs were then further clustered into OTUs, considering total read abundances, and using a maximum sequence divergence threshold of 3%, again with vsearch (Rognes et al., 2016). OTU abundances were obtained by mapping reads back to the representative OTU sequences.

Taxonomic classification was carried by aligning representative OTU sequences

to the SilvaMod database (v128) using blastn (v.2.2.25+ task megablast) and using the LCAClassifier of CREST with default parameters (Lanzén et al., 2012). Unclassified OTUs below the alignment threshold and those classified as belonging to eukaryotic organellar rRNA genes were excluded from further analysis. Statistical analyses were based on relative taxon abundances derived by CREST. For all analyses comparing community structure to physicochemical parameters, the two technical metabarcoding replicates at each site were pooled *in silico.*

8.3.6. Statistical analysis

A Cluster Analysis using squared Euclidean distance and Ward's method was performed to identify groups of sampling sites affected by similar contamination source (urban, industrial and untreated wastewater). Differences in gene abundance values for sediment samples and for water and surface sediment chemical parameters were estimated using one-way ANOVA and Tukey's range test. All variables were log-transformed and checked by Shapiro-Wilk test. Spearman correlation analysis was also performed. SPSS software for Windows 20.0 (SPSS, Inc) was used.

Diversity indices and rarefied richness were calculated based on metabarcoding OTU tables using R/vegan (Oksanen et al., 2013). Based on alpha diversity and relative taxon abundances, two-way ANOVA analyses and Tukey's range test were performed to establish significant differences among groups (type of contamination - residual, industrial or untreated wastewater- and river location). For continuous explanatory variables, Kendall's rank correlation was used. Bonferroni correction was always applied when evaluating significance.

Ordination and multivariate statistics based on pairwise community dissimilarities (Bray-Curtis) were performed using R/vegan. Non-metric multidimensional scaling (NMDS) was performed using *metaMDS*, fitting of variables to resulting NMDS space using *envfit*, variable selection using *bioenv,* and assessment of significance using specific models the function *adonis*. Permutation-based Mantel tests and partial Mantel tests were used to evaluate the correlation between dissimilarity matrices. Relative OTU abundances and Bray-Curtis dissimilarity was used to derive community dissimilarities, and for chemical parameters, Euclidian distance after standardization to an average of 0 and standard deviation to 1. Metal

concentrations included both pseudo-total metal concentration and hazard quotient (HQ) values.

Table 8.1. *Surface sediment and water river physicochemical parameters measured at each sampling site. ∑HQ: the sum of HQ values for all metals (Cu, Cr, Ni, Pb and Zn) considered. Different lowercase letters within a same parameter indicate that means (n = 3) are significantly different between samples at p < 0.05*

	Deba River						Ego stream	
	D1	**D2**	**D3**	**D4**	**D5**	**D6**	**E1**	**E2**
Surface sediments								
TOC (%)	2.75^{a}	1.84^{a}	2.64^{a}	1.59^{a}	1.85^{a}	1.28^{a}	1.89^{a}	1.65^{a}
TIC (%)	0.58^{a}	1.44^{a}	2.90^{ab}	1.73^{ab}	2.01^{ab}	1.48^{a}	0.50^{a}	4.04^{b}
TS (%)	0.08^{ab}	0.35^{c}	0.22^{bc}	0.02^{a}	0.15^{ab}	0.02^{a}	0.01^{a}	0.05^{b}
TOC:TIC	4.79^{a}	1.28^{b}	0.91^{bc}	0.92^{b}	0.92^{b}	0.87^{b}	3.75^{a}	0.41^{b}
TOC:TS	34.4^{a}	5.3^{a}	12.0^{a}	79.3^{a}	12.4^{a}	64.0^{a}	188.6^{b}	33.0^{a}
TON (%)	0.32^{ab}	0.19^{ab}	0.40^{b}	0.13^{a}	0.15^{a}	0.08^{a}	0.13^{a}	0.16^{ab}
NO_3^--N (mg kg^{-1})	6.0^{a}	6.0^{a}	14.6^{ab}	8.4^{a}	4.4^{a}	9.4^{a}	6.2^{a}	25.8^{b}
NH_4^+-N (mg kg^{-1})	9.0^{a}	4.3^{a}	20.1^{ab}	7.3^{a}	6.4^{a}	3.6^{a}	3.2^{a}	36.1^{b}
Zn (µg g^{-1})	170^{a}	261^{a}	288^{a}	373^{a}	407^{a}	406^{a}	148^{a}	306^{a}
Ni (µg g^{-1})	26.6^{ab}	32.0^{ab}	37.4^{ab}	45.9^{ab}	46.8^{ab}	68.0^{b}	18.5^{a}	38.7^{ab}
Cu (µg g^{-1})	15.5^{a}	59.6^{a}	60.7^{a}	58.9^{a}	66.1^{a}	51.3^{a}	13.3^{a}	69.6^{a}
Pb (µg g^{-1})	18.6^{a}	12.5^{a}	16.2^{a}	26.7^{a}	61.8^{b}	10.3^{a}	13.8^{a}	9.66^{a}
Cr (µg g^{-1})	21.6^{a}	51.5^{a}	28.7^{a}	44.0^{a}	63.9^{a}	56.9^{a}	33.5^{a}	41.5^{a}
∑HQ	0.21^{a}	0.18^{a}	0.39^{a}	0.27^{a}	0.20^{a}	0.20^{a}	0.21^{a}	0.28^{a}
River water								
pH	7.5^{a}	8.2^{a}	8.0^{a}	7.8^{a}	7.8^{a}	7.8^{a}	7.9^{a}	7.6^{a}
DOC (mg L^{-1})	1.8^{ab}	4.5^{ab}	5.0^{ab}	4.1^{ab}	4.0^{ab}	4.5^{ab}	1.2^{a}	5.9^{b}
POC (mg L^{-1})	0.2^{ab}	0.1^{ab}	3.0^{b}	0.1^{a}	2.2^{ab}	2.3^{ab}	1.9^{ab}	3.1^{b}
PO_4^{3-}-P (µg L^{-1})	1.4^{a}	10.3^{a}	543^{b}	130^{a}	155^{ac}	165^{ac}	7.9^{a}	428^{bc}
NO_3^--N (µg L^{-1})	140^{a}	305^{a}	5355^{b}	2103^{ab}	2539^{ab}	2273^{ab}	229^{a}	1722^{a}
NO_2^--N (µg L^{-1})	1.5^{a}	8.4^{a}	17.2^{a}	26.6^{a}	27.0^{a}	40.8^{a}	4.2^{a}	211^{b}
NH_4^+-N (µg L^{-1})	9.9^{a}	65.6^{a}	69.8^{a}	117^{a}	83.1^{a}	179^{a}	26.4^{a}	1388^{b}
Cl^- (mg L^{-1})	190.2^{a}	373.1^{b}	149.9^{a}	90.7^{a}	73.8^{a}	59.9^{a}	13.3^{a}	26.1^{a}
SO_4^{2-} (mg L^{-1})	84.5^{a}	223.3^{b}	132.3^{ab}	86.4^{a}	72.7^{a}	55.5^{a}	14.9^{a}	27.2^{a}
Alkalinity (meq L^{-1})	2.9^{a}	3.8^{a}	3.1^{a}	3.3^{a}	3.1^{a}	3.0^{a}	4.3^{a}	3.9^{a}
Ca^{2+} (mg L^{-1})	93.2^{a}	138.6^{a}	97.5^{a}	83.6^{a}	76.3^{a}	73.0^{a}	85.7^{a}	78.9^{a}
Na^+ (mg L^{-1})	93.9^{a}	196.6^{b}	84.5^{a}	51.4^{a}	41.2^{a}	36.8^{a}	6.7^{a}	14.0^{a}
Mg^{2+} (mg L^{-1})	12.1^{ab}	19.4^{b}	11.4^{ab}	8.3^{ab}	6.9^{a}	5.9^{a}	5.7^{a}	4.3^{a}
K^+ (mg L^{-1})	3.6^{a}	7.7^{ab}	11.1^{b}	6.4^{ab}	6.8^{ab}	7.0^{ab}	1.4^{a}	3.8^{ab}

A mixed model was used to identify taxa or diversity indices with differential relative abundance in samples taken upstream (D2, D4 and E1) *vs.* downstream (D3, D5, E2) of WWTPs or UWWs. Specific site (D2-D3, D4-D5 or E1-E2) was modelled as a random effect. Bonferroni correction was used to compensate for multiple testing. Taxon abundances were

compared at each rank individually and correction based on the number of taxa at that rank, but when abundance did not diverge between a taxon and its parent, only the taxon at the lowest level was reported.

8.4. Results

8.4.1. Chemical parameters

In surface sediments, the highest TOC:TIC ratios (Table 8.1) were found at headwaters (D1 and E1), while the lowest TOC:TS ratio was found at D2, suggesting that sulphate reduction down the oxygenated water column might be happening (Appelo and Postma, 2005). NH_4^+-N and NO_3^--N concentrations were highest in sediments downstream of the point of discharge of WWTP (D3) and UWW (E2) effluents. The highest and lowest pseudo-total metal concentrations were detected in the Deba River and Ego stream (from D2 to D6, and E2) and their headwaters (D1 and E1), respectively. Zinc was by far the most abundant metal in all the sites (Table 8.1). TOC correlated positively with TON ($\rho = 0.75$ at 0.01 level), suggesting that the main source of organic nitrogen is possibly organic matter. TIC also correlated positively with NH_4^+-N in surface sediments.

The pH in the river water varied between 7.5 and 8.2 (Table 8.1), indicating a weakly alkaline environment but well-buffered waters. Nutrient concentrations, as well as DOC and POC values, showed a trend of downstream increase, where the highest values were exhibited downstream of WWTP (D3 and D5) and UWW (E2) effluents. In water samples, PO_4^{3-}-P values correlated positively with values of all nitrogen forms determined here ($\rho > 0.74$ at 0.01 level), as well as with DOC values ($\rho = 0.83$ at 0.01 level), suggesting that the main source of these chemicals was likely residual (WWTPs) and untreated (UWW) effluents discharged in the water courses. Some metals (Zn, Ni, Cu) were positively correlated ($\rho > 0.71$ at 0.01 level) with nutrients (NO_3^--N, NO_2^--N, NH_4^+-N, PO_4^{3-}-P) in river water, suggesting that those sites affected by WWTP and UWW effluents are also impacted by metal contamination. Although the highest ∑HQ (the sum of HQ values for all considered metals) were observed at D3 and E2, the threshold of 1 was not exceeded at any sampling site (Table 8.1), suggesting that the surface sediments do not represent a human health risk. The highest concentrations of the

major ions (Cl^-, SO_4^{2-}, Na^+, Ca^{2+}, K^+ and Mg^{2+}) were registered from D1 to D3, especially at D2. Values of SO_4^{2-},Cl^-, Na^+, Mg^{2+} presented a strong correlation to one another (p > 0.91 at 0.01 level), indicating that groundwater circulation from evaporitic rocks was being discharged into the river water.

According to the clustering analysis of nutrient, organic/inorganic carbon, and pseudo-total metal concentration data from sediment and water samples, sampling sites were divided into groups depending on the source of contamination (Fig. 8.2). When all parameters, except for pseudo-total metal concentrations (Table 8.1), were included in such clustering, two groups were identified (Fig. 8.2A): the first group included those sites upstream of WWTP and UWW discharge points (non-impacted sites: D1, D2 and E1). The second group was further divided into two sub-groups: sites impacted by UWW effluents (E2) and sites impacted by WWTPs (D3 and D5: sites immediately downstream of WWTPs; D4 and D6: sites downstream of WWTPs, Fig. 8.1). In water samples, values of PO_4^{3-}-P and NO_3^--N were significantly different in non-impacted sites, compare to sites located downstream of WWTP and UWW discharge points (p < 0.05, one way ANOVA, Table 8.1). By contrast, the clustering analysis of pseudo-total metal concentrations revealed a slightly different pattern (Fig. 8.2B): Deba and Ego headwaters (D1 and E1) were grouped together, representing non-impacted sites. The second group clustered those sites affected by residual (D3, D4, D5, D6), industrial (D2) and untreated (E2) effluents, suggesting that metal contamination is affecting the entire catchment, except for the headwaters.

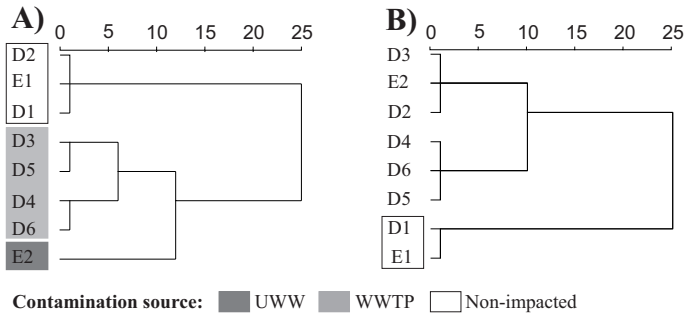


Fig. 8.2. *Cluster analysis using squared Euclidean distance and Ward linkage of the Deba River catchment sampling sites; A) dendogram of sampling sites based on surface sediment (TOC, TIC, TS, NO_3^--N, NH_4^+-N) and river water (DOC, POC, PO_4^{3-}-P, NO_3^--N, NO_2^--N, NH_4^+-N) chemical parameters; B) dendogram based on metal concentrations of surface sediment (Zn, Ni, Cu, Pb, Cr)*

Table 8.2. *Copy numbers of prokaryotic (16 rRNA) and denitrifying genes (nirK, nirS and nosZ) in surface sediments of each sampling site. The values (genes g^{-1} dry weight sediment) represent the mean ± standard error (n = 3). Different lowercase letters within a same parameter indicate that means are significantly different at $p < 0.05$; nir = nirK + nirS*

	16S rRNA ($x10^{14}$)	***nirS*** ($x10^{11}$)	***nirK*** ($x10^{9}$)	***nosZ*** ($x10^{5}$)	***nirS*:16S** ($x10^{-3}$)	***nirK*:16S** ($x10^{-5}$)	***nosZ*:16S** ($x10^{-9}$)	***nosZ:nir*** ($x10^{-7}$)	***nirK:nirS*** ($x10^{-3}$)
D1	1.85 ± 0.52^{abc}	3.29 ± 0.40^{a}	5.31 ± 0.65^{a}	2.44 ± 0.33^{a}	1.79 ± 0.07^{a}	2.89 ± 0.01^{a}	1.01 ± 0.07^{a}	5.62 ± 0.6^{a}	16.2 ± 0.08^{a}
D2	1.30 ± 0.17^{ac}	4.78 ± 0.06^{a}	5.53 ± 0.38^{a}	1.49 ± 0.01^{a}	3.73 ± 0.58^{bc}	4.25 ± 1.15^{ab}	1.17 ± 0.14^{ab}	3.10 ± 0.1^{b}	11.6 ± 1.3^{ab}
D3	3.52 ± 0.92^{d}	12.50 ± 0.50^{bcd}	4.91 ± 0.40^{a}	2.89 ± 0.19^{ab}	3.69 ± 0.66^{bc}	1.39 ± 0.14^{a}	0.75 ± 0.09^{a}	2.05 ± 0.1^{b}	3.92 ± 0.3^{c}
D4	1.42 ± 0.004^{abc}	5.96 ± 0.002^{a}	3.97 ± 0.03^{a}	1.52 ± 0.002^{a}	4.20 ± 0.007^{bcde}	2.80 ± 0.04^{a}	1.07 ± 0.006^{a}	2.54 ± 0.01^{b}	6.66 ± 0.1^{bcd}
D5	2.47 ± 0.30^{abd}	13.30 ± 0.90^{bce}	7.11 ± 1.90^{a}	3.14 ± 0.26^{ac}	5.36 ± 0.10^{e}	2.87 ± 0.12^{a}	1.08 ± 0.08^{a}	2.01 ± 0.1^{b}	5.35 ± 2.1^{bcd}
D6	0.95 ± 0.01^{ce}	5.29 ± 0.50^{a}	3.46 ± 0.07^{a}	1.48 ± 0.02^{a}	5.60 ± 0.10^{de}	3.66 ± 0.20^{a}	1.60 ± 0.02^{c}	2.85 ± 0.6^{b}	6.53 ± 0.9^{bcd}
D7	2.51 ± 0.07^{bd}	13.72 ± 2.90^{ce}	17.5 ± 1.25^{b}	3.90 ± 0.10^{ce}	5.50 ± 0.13^{de}	6.96 ± 0.52^{b}	1.56 ± 0.45^{bc}	2.80 ± 0.1^{b}	12.7 ± 6.1^{a}
E1	2.11 ± 0.19^{abe}	7.73 ± 0.80^{ad}	9.15 ± 2.70^{a}	5.20 ± 0.02^{ad}	3.63 ± 0.44^{c}	4.33 ± 0.21^{ab}	2.46 ± 0.21^{d}	6.61 ± 0.1^{a}	11.8 ± 4.6^{ad}
E2	3.35 ± 0.98^{d}	16.00 ± 2.70^{be}	5.60 ± 0.24^{a}	4.30 ± 0.36^{be}	4.72 ± 0.21^{bcde}	1.67 ± 0.37^{a}	1.12 ± 0.12^{a}	2.29 ± 0.4^{b}	3.51 ± 0.9^{c}

8.4.2. Prokaryotic community structure and alpha-diversity

Absolute abundances of 16S rRNA gene copies (estimated by qPCR) were higher at sites immediately downstream of WWTP (D3, D5) and UWW (E2) effluents, as well as at the stream bank sediment (D7), than at sites located downstream of WWTPs (D4 and D6) ($\rho < 0.05$, Table 8.2). Furthermore, the *nirS*:16S rRNA ratio correlated positively with NH_4^+-N and NO_2^--N concentrations in river water ($\rho > 0.81$ at 0.01 level), as well as with sediment Zn, Ni and Cr concentrations ($\rho > 0.83$ at 0.01 level). Instead, the *nirK* or *nosZ* to 16S rRNA ratio correlated negatively with sediment NH_4^+-N concentration ($\rho < -0.76$ at 0.01 level), suggesting that *nirS* denitrifiers were partly responsible for the increase in prokaryotic biomass in sites highly affected by residual (D3, D5) and untreated (E2) effluents, and that *nirK*- and *nosZ*-denitrifier populations were negatively affected by those effluents. The *nosZ:nirS* and *nirK:nirS* ratios were negatively affected ($\rho < -0.76$ at 0.01 level) by the presence of one or several metals (Zn and Cu) in surface sediments.

Table 8.3. *Reads and diversity overview based on 16S rRNA metabarcoding (pooled replicates)*

Sampling site	16S rRNA reads[a]	OTUs	Rarefied richness	Shannon (H')	Pielou evenness (J')
D1	97,319	7,506	7,108	7.28	0.82
D2	110,476	8,037	7,181	7.04	0.78
D3	85,101	5,680	5,680	6.46	0.75
D4	104,223	7,137	6,519	6.70	0.76
D5	99,564	5,977	5,589	6.43	0.74
D6	202,996	10,018	7,016	7.01	0.76
D7	105,379	6,389	5,805	6.31	0.72
E1	190,007	12,040	8,600	7.46	0.79
E2	93,354	5,169	4,960	5.95	0.70
Total	*1,088,419*	*22,606*			

[a] Number of overlapped read pairs after overlapping, quality filtering, OTU clustering and removal of putative chimeras.

All denitrifying genes (*nirS, nirK* and *nosZ*) were detected in all surface sediments but showing different distribution patterns (Table 8.2). The absolute abundance of *nirS* gene copies increased at sites immediately downstream of residual (D3 and D5) and untreated (E2) effluents, compared to headwaters (D1 and E1, $\rho < 0.05$) and to sites downstream of WWTPs

(D4 and D6, $\rho < 0.05$, Tables 8.3 and 8.4). Moreover, *nirS, nosZ* and 16S rRNA gene copy abundances were significantly correlated ($\rho < 0.70$ at 0.01 level), whereas *nosZ* and *nirK* gene abundances were also correlated ($\rho = 0.78$ at 0.01 level). The highest copy numbers of *nosZ* gene were detected at Ego headwater (E1), followed by the site downstream of UWW (E2), the stream bank sediment (D7), and the two sites located immediately downstream of WWTPs (D3, D5). Likewise, the lowest *nosZ*:16S rRNA ratio was registered immediately downstream of the WWTP (D3). The *nosZ:nir* ratio showed a clear difference between headwaters (D1, E1) and the rest of the sampling points, being significantly higher at headwaters ($\rho < 0.05$, Table 8.2). Finally, the *nirK*:*nirS* ratio was higher at sites non-impacted by residual effluents (D1, D2, E1) and at the stream bank sediment (D7), whereas the lowest *nirK:nirS* ratio was registered at sites impacted by residual (D3) and UWW (E2) effluents ($\rho < 0.05$, Table 8.2).

Table 8.4. *Parameters and taxon abundances differing significantly between sites inmediately upstream vs. downstream of WWTPs or UWW, according to linear mixed model*

Parameter or taxon	Maximum	ρ-value[a]
16S Rarefied richness	Upstream	$2.8E^{-03}$
16S Shannon diversity	Upstream	$1.3E^{-02}$
16S Pielou evenness	Upstream	$4.9E^{-02}$
nirS copies	Downstream	$2.4E^{-02}$
Gammaproteobacteria (class)	Downstream	$8.3E^{-03}$
Holophagae (class)	Upstream	$6.8E^{-03}$
Methylophilales (order)	Upstream	$4.8E^{-02}$
Syntrophobacterales (order)	Upstream	$4.0E^{-02}$
Alteromonadaceae (fam.)	Downstream	$7.5E^{-03}$
Hydrogenophilaceae (fam.)	Upstream	$2.4E^{-02}$
Rhodocyclaceae (fam.)	Downstream	$2.7E^{-02}$
Sphingomonadaceae (fam.)	Upstream	$3.9E^{-02}$
Spirochaetaceae (fam.)	Upstream	$2.0E^{-02}$
Sulfuricurvum (genus)	Upstream	$8.2E^{-03}$
Thiobacillus (genus)	Upstream	$1.7E^{-02}$

[a] ρ-Values given after Bonferroni correction (multiplied with number of taxa at current rank).

Amplicon sequencing resulted in just above one million high quality classified 16S rRNA gene reads (85,101 – 202,996 per sample) clustered into 22,606 OTUs (Table 8.3). As opposed to 16S rRNA gene abundance, prokaryotic diversity estimates including rarefied richness and Shannon (H') were significantly lower at sites impacted by WWTP (D3, D5) and UWW (E2) effluents, compared to non-impacted sites (D1, D2, E1). This is consistent

with sites located upstream of WWTPs and UWW being significantly more diverse (higher OTU richness, H' and evenness) compared to those downstream (Table 8.4). Further, H' was negatively correlated with TIC.

8.4.3. Influence of chemical parameters on community dissimilarity structure

NMDS analysis of 16S rRNA gene data (Fig. 8.3) indicated that community composition depended on the level of nutrients (NO_3^--N and PO_4^{3-}-P) and DOC in the river water, as well as sediment Cu levels and the TOC:TIC ratio. The two headwater samples (D1, E1) formed a separate cluster, distinct from all samples affected by residual and untreated effluents (WWTPs and UWW). The *nosZ*:*nirS* ratio also correlated with community dissimilarities, as indicated by NMDS. In addition, the *nosZ*:*nirS* ratio was negatively correlated with PO_4^{3-}-P and NO_3^--N concentrations in river water , and with TIC and some metal (Cu and Zn) concentrations in surface sediments ($\rho < -0.76$ at 0.01 level). However, organic and inorganic carbon (DOC, POC, TIC), water nutrient concentrations (PO_4^{3-}-P, NO_3^--N, NO_2^--N, NH_4^+-N) and sediment Cu concentration negatively correlated ($\rho < -0.76$ at 0.01 level) with values of the *nirK*:*nirS* ratio, whereas the TOC:TIC ratio correlated positively ($\rho = 0.88$ at 0.01 level).

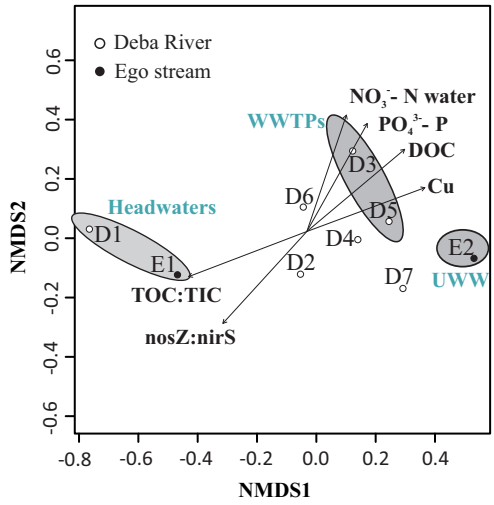


Fig. 8.3. *Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities of microbial community composition. Composition based on Hellinger-transformed relative OTU abundances from prokaryotic 16 rRNA. Black vectors indicate fitted chemical and biological (nosZ:nirS) parameters significantly correlated to NMDS coordinates*

The significant influence of Cu, TOC:TIC and contamination sources (according to the clustering analysis, Fig. 8.2A) on community structure was confirmed by PERMANOVA in addition to TIC (Table 8.S2). Although industrial contamination level (Fig. 8.2B) and individual nutrient concentrations did not result in significant PERMANOVA models, the combined influence of nutrients and pseudo-total metal concentrations correlated significantly with community structure according to Mantel test (Table 8.S3). Moreover, metal concentrations influenced community structure independently of nutrients, although industrial and residual contamination levels were highly correlated.

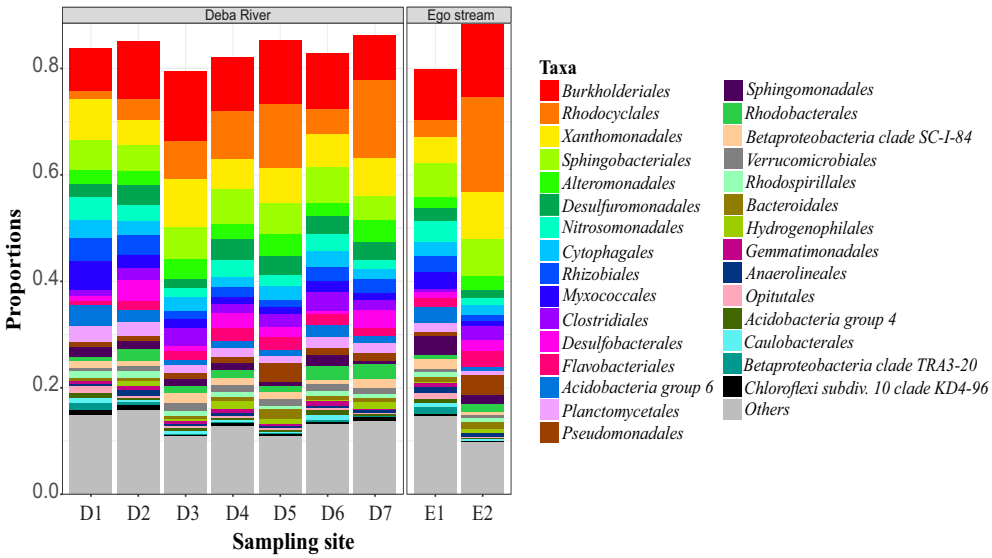


Fig. 8.4. *Distribution of most abundant taxa across samples. Relative abundances of taxa at order level are presented as bar-charts of each individual sampling site. A significant portion of all classified OTUs could only be classified to higher taxonomic levels (domain, phylum or class). Thus, relative abundance at order level does not sum up to 100%*

8.4.4. Taxonomic community composition and influence of contamination and other parameters on individual taxa

In average, 84% of all high-quality 16S rRNA gene reads could be classified to order rank or lower. The most abundant taxon at order level was *Burkholderiales* (class *Betaproteobacteria*), followed by *Rhodocyclales* (*Betaproteobacteria*), *Xanthomonadales* (*Gammaproteobacteria*) and *Sphingobacteriales* and *Alteromonadales* (*Gammaproteobacteria*) (Fig. 8.4). Although

not significant, the relative abundance of *Rhodocyclales*, which contains many denitrifiers, increased along the trajectory of both rivers (Deba River and Ego stream).

Gammaproteobacteria as well as the families *Alteromonadaceae* and *Rhodocyclaceae* were significantly more abundant at sites immediately downstream of WWTP or UWW effluents compared to upstream (Table 8.4). Although not significant, several genera belonging to the *Rhodocyclaceae* showed clear differences in upstream *vs.* downstream relative abundance (Table 8.S4), including five genera previously linked to WWTP or UWW contaminated sediments.

Several taxa instead showed the opposite trend, being less abundant immediately downstream of WWTP and UWW effluents: *Holophagae* (a class of *Acidobacteria*), *Syntrophobacterales* (an order of *Deltaproteobacteria* including many sulphate-reducers), *Methylophilales* (an order of mainly methylotrophic *Betaproteobacteria*), *Spirochaetaceae*, *Sphingomonadaceae* (a family of *Alphaproteobacteria*), *Hydrogenophilaceae* (a family of *Proteobacteria*), *Sulfuricurvum* (a genus of facultatively aerobic chemolithoautotrophic sulphur-oxidising *Epsilonproteobacteria*) and *Thiobacillus* (a genus of autotrophic sulphur-oxidising *Betaproteobacteria*).

8.5. Discussion

The spatial-distribution and concentration of nutrients, organic and inorganic carbon and metals in river water and surface sediments evidence (i) the non-contaminated nature of the Deba and Ego headwaters and (ii) the contamination of the mid- and low-water courses of the Deba River catchment, similar to what has been observed for many urban river ecosystems (Spänhoff et al., 2007; Steele et al., 2010). WWTPs are designed to meet regulatory guidelines by means of the application of mechanical, chemical and/or biological treatments (Weisener et al., 2017). However, WWTPs are not always effective and, in consequence, their effluents can have an adverse impact on river water and sediment quality in those sites located immediately downstream of WWTPs (e.g., D3 and D5), but also in more downstream sites (e.g., D4 and D6). In our study, both treated and untreated wastewater effluents had remarkably similar effects on the chemical properties of the Deba River catchment, with respect to their capacity

to reduce the natural chemical quality of the river.

Certain environmental factors (e.g., nutrients and carbon load) are known to impact the composition, spatial-distribution and abundance of microbial communities in river surface sediments (Wakelin et al., 2008; Weisener et al., 2017; Yu et al., 2017). Our results show that some chemical characteristics of the river water (PO_4^{3-}, NO_3^- and DOC) and surface sediments (TOC:TIC and Cu, Zn and Ni concentrations) have the most impact on the abundance and spatial-distribution of sediment bacterial communities. Based on the high nutrient and organic carbon concentrations found in the Deba River catchment, it is not surprising that higher 16SrRNA gene abundances of sediment bacterial communities were detected at sites immediately downstream of WWTP and UWW effluents, with *nirS*-type denitrifiers as the major contributor to denitrifying communities (as reflected by the lowest *nirK:nirS* and *nosZ:nirS* ratios) in those contaminated sites. On the contrary, prokaryotic diversity (rarefied richness and Shannon H') decreased significantly downstream of WWTP and UWW effluents discharge. The fact that WWTP effluents were neither chlorinated nor filtered before being discharged into the river suggests that *nirS*-type denitrifier communities already present in the residual effluents might have ended up in surface sediments, so that *nirS* gene abundance at D3 and D5 sites was of the same order of magnitude to that observed in sites affected by UWW effluents (E2) and four times higher than that detected at the Deba headwater (D1).

The observed spatial-distribution changes in the abundance of *nir*-type denitrifiers could possibly be linked with changes in environmental and hydrological factors. Several studies have demonstrated that *nirS*-type denitrifiers prefer more anoxic environments than *nirK*-type denitrifiers, the latter not being as sensitive to oxygen (Desnues et al., 2007; Knapp et al., 2009; Wang et al., 2016). A higher predominance of *nirK* over *nirS* gene was observed at the stream bank sediment sample (D7, Table 8.2). The greyish colour of this sediment indicates the presence of anoxic conditions, but its location in the stream bank suggests that it was transported and deposited under more aerobic conditions after flooding. This would explain the lower values of the *nirK:nirS* ratio found in the stream bed sediment (D6), compared to the stream bank sediment (D7). This fact reflects the importance of always taking into consideration the chemical and biological heterogeneity of river sediments when designing a sediment sampling scheme.

The correlation observed here between the abundance of *nirS*- and *nirK*-type denitrifiers with the abundance of the *nosZ* gene points out to the existence of denitrifying communities capable of carrying out a complete denitrification in all studied sites. However, the significant decrease in the *nosZ:nir* ratio (Table 8.2) observed in sites impacted by residual (WWTP) and untreated (UWW) effluents, and to a lesser-extent also in sites impacted only by IWW (D2), compared to non-impacted sites (headwaters), points out to the predominance of incomplete denitrification in impacted sites. Philippot et al. (2013) demonstrated that a lower microbial diversity, such as that observed here in sites impacted by WWTP and UWW effluents, can result in a lower potential of complete denitrification activity, thus favouring N_2O emissions.

In contrast, higher values of the *nosZ:nir* and *nirK:nirS* ratios might explain the predominance of *nosZ*- and *nirK*-type denitrifiers over *nirS*-type denitrifiers at Deba River and Ego stream headwaters (non-impacted sites). The higher abundance of *nosZ* over *nir* genes suggests that complete denitrification might be occurring in non-impacted sediments characterized by a lower nutrient level. Many studies have linked complete denitrification with the abundance of the *nosZ* gene in low-nutrient (Huang et al., 2011) or high-nutrient sediments (Laverman et al., 2010). Nevertheless, our results demonstrate that although the abundance of the *nosZ* gene can be high in both low- and high-nutrient environments, a lower potential to emit N_2O was detected in low-nutrient sites, as reflected by the higher values of the *nosZ:nir* ratio. Although gene expression studies are required for a suitable identification of those genes contributing more to denitrification (Yoshida et al., 2009), our qPCR data already indicate that the release of nutrient-rich wastewater effluents into rivers alters nitrogen cycling pathways and affects the composition and spatial-distribution of sediment bacterial communities.

In the Deba River catchment, the main sources of metal contamination are industrial and residual (urban and industrial) effluents. This metal contamination is affecting mid- and low-water courses of the Deba River (from D2 to D6) and Ego stream (E2). The presence of high metal and nutrient concentrations, both singly and in combination, influenced the structure of sediment bacterial communities. The abundance of *nirK*- and *nosZ*-type denitrifiers appeared to be more affected by the presence of industrial (Cu and Zn concentrations) and wastewater (nutrients and inorganic carbon) effluents than the abundance of *nirS*-type denitrifiers,

suggesting that *nirK* and *nosZ*-type denitrifiers are more sensitive to contamination, as reported by Magalhães et al. (2011) and Ligi et al. (2014). Although the relative abundance of *nosZ* and *nirK* genes, with respect to the *nirS* gene, appeared more affected by metal concentrations (Cu and Zn for *nosZ*, and Cu for *nirK*), this effect might have arisen by a covariate correlation between metal and nutrient concentrations, since industrial and residual contaminations were extremely correlated in the Deba River catchment (except for the headwaters).

Significant differences in the abundance of *Alteromonadaceae* and *Rhodocyclaceae* were found downstream of WWTPs and UWWs, compared to upstream sites. Most members of *Alteromonadaceae* are chemoorganotrophic and many have the ability to reduce nitrate (López-Pérez and Rodriguez-Valera, 2014). The presence of *Alteromonadaceae* in WWTP and UWW effluent-impacted sites reflects their high nutrient levels (Yu et al., 2017). Several of the genera in the *Rhodocyclaceae* family detected at higher concentration downstream of WWTP and UWW effluents have also been previously associated with residual contamination sources, including *Thaurea*, *Candidatus Accumulibacter*, *Denitratisoma* and *Ferribacterium. Thauera* has been frequently detected in wastewater impacted environments and appears to play a key role in partial denitrification (Du et al., 2017a, 2017b; Liu et al., 2013). The uncultured type strain of *Candidatus Accumulibacter* (*A. phosphatis*) is commonly found in WWTPs and can perform phosphorus removal (Seviour et al., 2003). The type species of *Denitratisoma* is a denitrifier isolated from activated slugde (Fahrbach et al. 2006). The only known species of *Ferribacterium*, finally, is a Fe-reducing obligate anaerobe, isolated from mining-impacted freshwater lake sediments (Cummings et al. 1999). The higher abundance of those genera in the WWTP and UWW impacted sites, reflects the effect of treated and untreated effluents on the surface sediment bacterial community structure in the Deba River catchment.

The high SO_4^{2-} concentrations in river water, together with the low TOC:TS ratios in surface sediments located in the Deba River headwater, points out to the fact that biological sulphate reduction might be occurring under these conditions. This fact is supported by an increased abundance of the order *Syntrophobacterales*, which includes many sulphate-reducing bacteria, in sediments non-impacted by residual wastewater (D1 and D2) located in the Deba River. Most described species of the family *Syntrophobacteraceae* are strictly anaerobic che-

moorganoheterotrophic sulphate-reducers (Kuever, 2014), thus leading hydrogen sulphide gas to react with many divalent transition metals to form very insoluble metal sulphides (Fu and Wang, 2011). This is corroborated by the precipitation of Fe-, Zn-, Ni- and Cu-sulphides previously reported in surface sediments from the Deba River headwater (Martínez-Santos et al., 2015). Moreover, the significantly higher abundance of S-reductive *Syntrophobacterales* and C-degradative *Holophagae* (Acidobacteria) in anaerobic sediments of the Deba River headwater points out to an interaction between sulphur and carbon cycles, as reported by Elshahed et al. (2003) in a sulphide-rich spring. The presence of *Sphingomonadaceae* might be indicative of the good habitat quality of the Deba River headwater, since this family is known to be adapted to oligotrophic environments and involved in the maintenance of ecosystem health (Wakelin et al., 2008).

The sulphur-oxidizing genera *Sulfuricurvum* and *Thiobacillus* were also more abundant in sediments non-impacted by residual wastewater (D1 and D2) located in the Deba River. In sediments subjected to anoxic conditions, the presence of *Sulfuricurvum*, in which *S. kujiense* has been identified as a facultative sulphur-oxidizer (Kodama and Watanabe, 2004), suggests that oxygen-mediated sulphide oxidation was limited, being nitrate the most likely electron acceptor. *Thiobacillus* is another well-known sulphide-driven autotrophic denitrification genus (Pokorna and Zabranska, 2015), which uses reduced forms of sulphur while simultaneously reducing nitrate to nitrogen gas. The presence of both genera at the Deba River headwater indicates that autotrophic denitrification is the predominant nitrogen pathway. Although sulphide-driven autotrophic denitrification can certainly be a potential source of N_2O emissions (Burgin et al., 2008), Yang et al. (2016) reported the high efficiency of this process to complete the nitrogen cycle (i.e., complete denitrification from NO_3^- to N_2).

Finally, the combination of functional qPCR and structural (16S rRNA gene) metabarcoding has proved to be very useful for the detection of the effects of anthropogenic disturbances on nitrogen and sulphur cycles, as well as on the spatial-distribution, diversity and composition of river sediment bacterial communities. As shown in Fig. 8.5, in the Deba River headwater (sediments non-impacted by residual wastewater: D1 and D2), coupled sulphate-reducing (*Syntrophobacterales*) and sulphur-oxidizing (*Thiobacillus* and *Sulfuricurvum*) processes may simultaneously coexist in surface sediments, which supports

the previous observations of Cao et al. (2017) in freshwater sediments. The higher abundance of those sulphide-driven autotrophic denitrification genera, together with the presence of metal-sulphides, indicates that autotrophic denitrification might be the predominant nitrate removal pathway in headwater sediments. Moreover, the high absolute and relative (*nosZ:nir*) abundance of *nosZ*-type denitrifiers suggests that complete denitrification is occurring in non-impacted sediments. On the other hand, anthropogenic disturbances (WWTP and UWW effluents) can impact biogeochemical cycles, resulting in partial heterotrophic denitrification being the predominant nitrate removal pathway in sites located immediately downstream of WWTP and UWW effluents, resulting in potentially higher values of N_2O emissions (a lower *nosZ:nir* ratio).

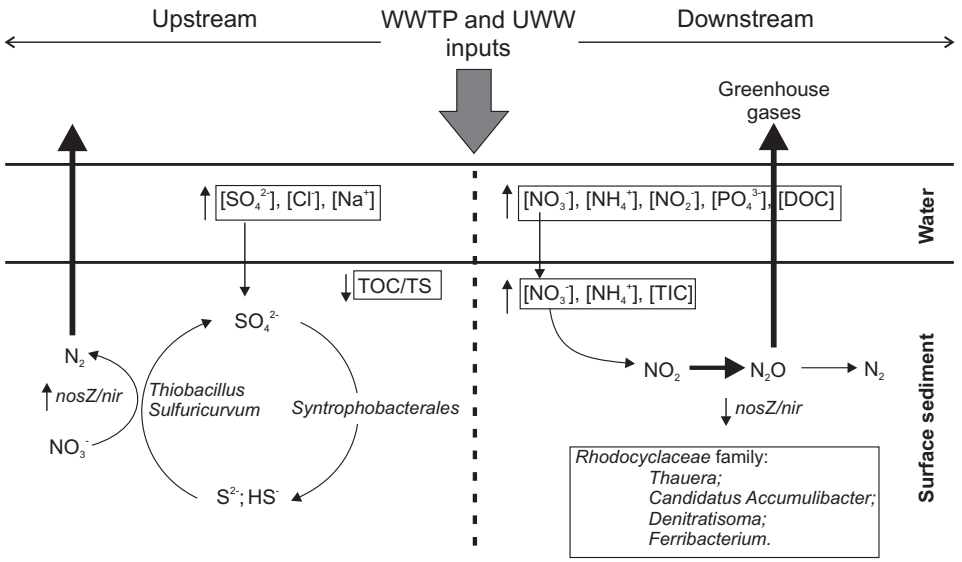


Fig. 8.5. *Schematic model showing changes in nitrogen and sulphur cycles and green house gases emissions as a function of differences on the distribution, structure and abundance of microbial communities up- and downstream of WWTP and UWW effluents. Arrow thickness represent the preferential gas emission (N_2O or N_2) given by the relative contribution of nosZ to nir (nirS + nirK) genes (Table 8.3)*

8.6. Conclusions

Studying the dynamics of nitrogen and sulphur cycling bacteria in river surface sediments is essential to better understand their contribution to global biogeochemical cycles. The combination of functional qPCR and structural (16S rRNA gene) metabarcoding has

proved to be a suitable approach to predict changes in river ecosystem functions (nitrogen and sulphur cycling) and sediment bacterial communities. The saline springs inflow to river waters, characterized by high sulphate concentrations, appears to favour the coexistence of bacteria involved in both sulphur reduction (*Syntrophobacterales*) and sulphur oxidation. Sulphide-oxidation coupled to nitrate reduction (*Thiobacillus* and *Sulfuricurvum*), instead of heterotrophic denitrification, seems to be the predominant denitrification process in those sites non-impacted by residual (urban and industrial) effluents. The discharge of WWTP and UWW effluents into the Deba River catchment altered the spatial-distribution, diversity and composition of sediment bacterial communities involved in sulphur and nitrogen transformations, favouring incomplete heterotrophic denitrification (a lower *nosZ:nir* ratio), as opposed to the autotrophic denitrification observed here in sediments non-impacted by residual wastewater, thus favouring N_2O emissions.

8.7. References

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8.8. Supplementary material

Table 8.S1. *Primer sets used for target gene amplification in qPCR and metabarcoding*

Target gene	Primer	Sequence (5' - 3')	References
16S rRNA gene metabarcoding			
16S rRNA	519F	CAGCMGCCGCGGTAA	Adapted from Øvreås et al., 1997
	806R	GGACTACHVGGGTWTCTAAT	D'Amore et al., 2016
qPCR			
16S rRNA	1055F 1392R	ATGGCTGTCGTCAGCT ACGGGGCGGTGTGTAC	Huang et al., 2011
nirK	F1aCu R3Cu	ATCATGGT(C/G)CTGCCGCG GCCTCGATCAG(A/G)TTGTGGTT	Deslippe et al., 2014
nirS	cd3aF R3cd	GT(C/G)AACGT(C/G)AAGGA(A/G)AC(C/G)GG GA(C/G)TTCGG(A/G)TG(C/G)GTCTTGA	Throbäck et al., 2004
nosZ	Nos661F Nos1773R	CGGCTGGGGGCTGACCAA ATRTCGATCARCTGBTCGTT	Braker et al., 2011

Table 8.S2. *Permutational multivariate analysis of variance using distance matrices (PERMANOVA). Only models where all variables were evaluated as significant (according to F-tests) are listed. Asterisks indicate the least significant explanatory variable (** < 0.01, * < 0.05). Sampling sites impacted by wastewater treatment plant (WWTP) and untreated wastewtaer (UWW) effluents. Chemical parameters: total inorganic and organic carbon (TIC and TOC) and Cu concentration*

Samples	Explanatory variable	Residual/total degrees of freedom	R^2 (16S rRNA)
All	Location	6/8	0.47**
All	Residual contamination level (from WWTPs or UWWs)	6/8	0.41*
All	Non-impacted *vs*. Impacted (WWTPs or UWWs)	7/8	0.33**
All except D7	TIC	6/7	0.35*
All except D7	TOC:TIC	6/7	0.42**
All except D7	(1) Total inorganic carbon (2) Non-impacted *vs*. Impacted	5/7	0.55*
All except D7	Cu	6/7	0.41**

Table 8.S3. *Results from Mantel tests and partial Mantel tests evaluating the influence of heavy metals and nutrients on bacterial community structure*

Explanatory variables	Conditioning variables	Dependent variables	R statistic	Significance
Metal concentrations	(None)	Prokaryotic comunity composition	0.60	$\rho = 0.002$
Nutrient concentrations	(None)	Prokaryotic comunity composition	0.60	$\rho = 0.01$
Metal concentrations	Nutrient concentrations	Prokaryotic comunity composition	0.45	$\rho = 0.02$

Table 8.S4. *Average abundance of genera of the family Rhodocyclaeceae across all samples, and inmediately upstream or downstream of WWTP or UWW. Only genera with average abundance > 0.01% are included*

Genus	All samples	Upstream	Downstream
Thauera	0.05%	0.04%	0.06%
Ca. Accumulibacter	0.27%	0.22%	0.60%
Uliginosibacterium	0.16%	0.19%	0.13%
Denitratisoma	0.08%	0.06%	0.22%
Propionivibrio	0.17%	0.24%	0.36%
Ferribacterium	0.07%	0.04%	0.24%

9

Results and discussion IV

Multivariate statistical analysis for water and sediment quality index development: a study of susceptibility in an urban river

Journal Pre-proofs

Multivariate statistical analyses for water and sediment quality index development: a study of susceptibility in an urban river

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9. Multivariate statistical analyses for water and sediment quality index development: a study of susceptibility in an urban river

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9.1. Abstract

In this study, multivariate statistical analyses were performed to develop water and sediment quality indexes, allowing us (i) to select with reliability the most appropriate chemical variables for the evaluation of river quality susceptibility; (ii) to weight the influence of each variable based on monitored data; (iii) to consider possible synergism or antagonism derived from the combined effect of several pollutants; and (iv) to express the quality as a deviation from selected site-specific reference conditions. For the establishment of these

threshold/maximum values, combining two biological indicators related to denitrifying bacteria in sediments turned out to be applicable to ensure compliance with the European water quality standard.

The joint implementation of water and sediment quality indexes assisted us in the rapid detection of the deleterious effect of different anthropogenic contamination sources, as well as the influence of hydrological regime seasonality on river quality. In addition, metal-dependent water quality appeared to be coupled to sediment dynamics, since they were preferentially adsorbed onto sediments during low flow seasons, whereas there was potential for metal mobilization to water during sediment resuspension in high flow seasons. Therefore, an annual determination of sediment quality index was also recommended as suitable tool for prospective monitoring water quality, identifying those sites which could deserve special attention during certain periods, and planning future strategies for river quality improvement. However, two limitations were found: (1) sediment was not appropriate for water physicochemical quality early monitoring due to organic matter and nutrient continuous transformation; and (2) a multimetric index did not provide a concise and definitive quality information, thus a new tool for combining with quality index was proposed for specifically evaluate the water and sediment quality by identifying pollutant/s of concern at each location.

9.2. Introduction

Water quality indexes have become a valuable tool for assessing river quality, since they transform multiple environmental indicator measurements into a single dimensionless number. As a result, they are used to assist water authorities, policy makers and the general public in rapidly detecting spatial and temporal trends, identifying pollutant sources, assessing regulatory policies and environmental programs, and making recommendations for future improvements (Yisa and Jimoh, 2010; Finotti et al., 2015; Gitau et al., 2016).

Although many water quality indexes have been developed and applied around the world, there is still no commonly accepted methodology for developing the index. Generally speaking, they entail four steps: (1) selection of indicators; (2) obtention of sub-indexes or transformation of measured values to a common scale; (3) assignment of weights; and (4)

aggregation of the weighted sub-index to compute the final index value (Sutadian et al., 2016). Typically, the indexes most widely used at global level (e.g. National Sanitation Foundation Water Quality Index (Brown et al., 1970), Scottish Research Development Department Index (SRDD, 1976), House's Water Quality Index (House, 1986) or Oregon Water Quality Index (Cude, 2001)), rely on expert judgement by implementing the Delphi technique for the first three steps. This procedure is used to obtain the most reliable consensus of opinion of a group of specialists by a series of intensive questionnaires interspersed with controlled feedback (Dalkey and Helmer, 1963), thus introducing a degree of subjectivity into the process. However, the application of multivariate statistical techniques, such as Principal Component Analysis (PCA), may help increase the level of objectivity and certainty. This method offers the possibility of introducing historical data monitored at different locations throughout the study area, and the maximum gradients observed are used to weight the influence of each indicator on water quality (URA, 2008; Primpas et al., 2010; Selle et al., 2013). As required by the European Water Framework Directive (WFD, 2000/60/CE), the classification of the ecological status of a river should be perform evaluating initially the biological elements and, ultimately, the chemical, physicochemical and hydromorphological elements supporting the biological ones. Therefore, the establishment of a reference conditions representing the maximum gradient of the selected chemical quality variables between the best and the worst ecological status is another essential step, and it should be based on the information provided by the biological quality assessment of the river.

For the purposes of evaluating river quality, sediments are also considered valuable quality indicators because of their capacity continuously to accumulate pollutants (Devesa-Rey et al., 2010; Bartoli et al., 2012). Their transport along the river is also coupled with sediment dynamics, where the deposition of suspended particles favours the accumulation of pollutants on the riverbed and sediment resuspension, usually occurring during flood events, promotes their mobilization (Rügner et al., 2014; Herrero et al., 2018).

To date, numerous indexes have been used to estimate the quality of river sediments, including the Geoaccumulation Index (Igeo) (Nowrouzi and Pourkhabbaz, 2014), Enrichment Factor (EF) (Kaushik et al., 2009), Potential Ecological Risk Index (PERI) (Kabir et al., 2011) and the Pollution Load Index (PLI) (Banu et al., 2013). However, these are not based

on matching chemical and biological data (Birch, 2018). On the other hand, the so-called Sediment Quality Guidelines (SQGs) —such as Effects Range Low and Median (ERL/ERM) (Tokatli, 2017), Predicted and Threshold Effects Level (PEL/TEL) (Zheng et al., 2008) and Ecological Risk Factor (ERF) (Kabir et al., 2011)— have been developed from empirical and mechanistic approaches, and used for individual chemicals or a mixture of substances to screen for contaminants that pose a risk to benthic communities (Birch, 2018). However, they are purely additives and do not consider possible synergism or antagonism derived from the combined effect of several pollutants.

The overall aim of this study is therefore to develop a new multimetric index for both water and surface sediments for a more reliable evaluation of the ecological status of the Deba River catchment. For this purpose, multivariate statistical analyses were used (1) to select the most appropriate chemical variables; (2) to consider any possible synergic/antagonist effect; and (3) to express the chemical quality as a deviation from reference conditions derived from biological quality evaluation. The specific objectives were (i) to identify sources of anthropogenic contamination; and (iii) to evaluate the influence of hydrological regime seasonality on the physical mechanisms governing pollutant exchange between water and surface sediments. We hypothesized that a combined analysis of water and surface sediment quality would provide us more comprehensive and detailed information on the susceptibility of a catchment that is subjected to multiple human pressures.

9.3. Materials and methods

9.3.1. Description of study area and sampling

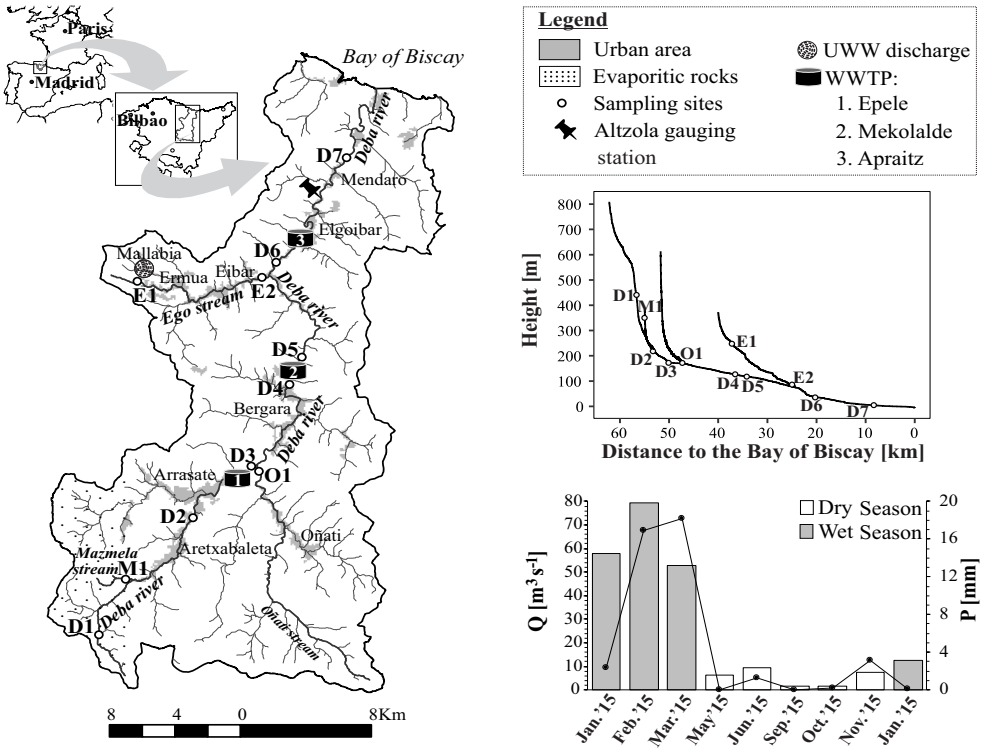
Historically, the Deba River catchment (538 km^2, Fig. 9.1) has been notable for its major ecological deterioration, caused by a dense population and intense industrial development, leading to discharges of metals, nutrients and/or organic-rich compounds into the main river and streams (Martínez-Santos et al., 2015). Until the recent construction of a sewerage system and the commissioning of three wastewater treatment plants (WWTPs) viz. the Apraitz (2007), Mekolalde (2008) and Epele (2012), ecological potential was reported as being moderate, poor or even bad in the main river and in several streams such as the Oñati

and the Ego (URA, 2013). Nonetheless, in recent years, there have been improvements in the degree of compliance with environmental targets (2013-2017), especially in the mid-high part of the main river and the Oñati stream. Even the Ego stream, which continues to be in serious breach of all biological indicators, recorded a slight positive change for the invertebrate community and fish fauna in the lastest campaigns (URA, 2018), probably as the result of the construction of a sewer from Ermua-Eibar to the Apraitz WWTP, which came on line in June 2014. Untreated urban wastewaters (UWW) from Mallabia are still being discharged into the Ego stream (Fig. 9.1).

According to meteorological and hydrological data measured and recorded every 10 minutes since October 1995 at the Altzola gauging station by the Provincial Council of Gipuzkoa (www.gipuzkoa.eus/es/deba), mean annual precipitation (P) and discharge (Q) for the last ten years (2008-2018) came to 1245 mm and 12.3 m^3 s^{-1} respectively. The wettest period is from January to March and the driest months is usually from July to October. Two different sets of hydrological conditions were therefore established for the research year (from Jan. 2015 to Jan. 2016): a high flow or wet season corresponding to sampling campaigns with Q values of over 12 m^3 s^{-1} (Jan. 2015, Feb. '15, Mar. 2015 and Jan. 2016); and a low flow or dry season comprising months with Q values of below 12 m^3 s^{-1} (Fig. 9.1).

In order to study the influence of different anthropogenic contamination sources on the river water and surface sediment quality, eleven locations along the Deba River catchment were chosen: in the main river (D1-D7), and in the Mazmela (M1), Oñati (O1) and Ego (E1-E2) tributaries (Fig. 9.1). Monthly or bimonthly water samples were collected in polyethylene bottles and water electrical conductivity (EC) was measured *in situ with* a Crison EC-Meter Basic 30+. By contrast, surface sediment sampling was exclusively carried out in the October 2015 campaign, a low flow month (1.435 m^3 s^{-1}; Fig. 9.1), in an attempt to diminish the influence of the hydrological pattern on the chemical and, predominantly, biological characteristics. As per the Environmental Protection Agency (USEPA, 2001), surface sediment subsamples (0-5 cm depth) from multiple points within each sampling location were collected using a sterilized spoon (hand implement recommended by Simpson et al., (2005) for areas exposed to low tide), sieved through a 2 mm mesh, composited in the field and sealed in sterile polypropylene containers. All water and surface sediment samples

were refrigerated in the dark and transported to the Chemical and Environmental Engineering laboratory (University of the Basque Country) on the same day.



Site	River/stream	Location	Contamination source	Sampling	
				Water	Sediment
D1	Deba	Headwater	Non-impacted	x	x
D2	Deba	Mid-water course	Industrial	x	x
D3	Deba	Mid-water course	WWTP + Industrial	x	x
D4	Deba	Mid-water course	WWTP + Industrial	x	x
D5	Deba	Mid-water course	WWTP + Industrial	x	x
D6	Deba	Low-water course	UWW + Industrial	x	
D7	Deba	Low-water course	WWTP + UWW + Industrial	x	x
M1	Mazmela	Low-water course	Non-impacted	x	
O1	Oñati	Low-water course	Industrial	x	
E1	Ego	Headwater	Non-impacted	x	x
E2	Ego	Low-water course	Urban + Industrial	x	x

Fig. 9.1. *Location of sampling sites, main urban areas, Altzola gauging station, untreated urban wastewater (UWW) discharge, and wastewater treatment plants (WWTPs) in the Deba River catchment. The table shows a summary of sampling types and the most important information from the sampling sites: location in the catchment and the impact of different contamination sources at each site (based on the results obtained in the spatial distribution of quality index (QI) in Fig. 9.5). The height profile of the catchment, and evolution of water discharge (Q; bar graph) and precipitation (P; lineal graph) during the research period are also included*

9.3.2. Analysis of environmental variables

Based on previous studies in the Deba River catchment (Martínez-Santos et al., 2018; Unda-Calvo et al., 2019a), focused on evaluating the involvement of multiple environmental variables in the ecosystem functioning, the following ones were analysed in water and surface sediment samples for the assessment of human impact on river quality. According to WFD (2000/60/CE) requirements for ecological status evaluation, they were grouped into chemical (physicochemical variables: salinity, alkalinity and nutrient concentrations; and specific pollutants: metals) and biological variables (related to activity and biodiversity of the denitrifying community in surface sediments). The biological variables in surface sediments were only used in the establishment of reference conditions for indexes development.

Analysis of chemical variables. In the laboratory, the water samples were filtered through a 0.45 µm Millipore nitrocellulose filter. One replicate of each sample was acidified to pH < 2 with HNO_3 (69%) for analysis of cations (Ca^{2+}, Mg^{2+}, Na^+ and K^+) and metals (Cu, Zn, Cr, Ni and Pb), using ICP-OES (Perkin Elmer Optima 2000) and an Ultrasonic Nebulizer (CETAC, U5000AT+; only for metals). Dissolved organic carbon (DOC) and anions (NO_3^-, SO_4^{2-} and Cl^-) were measured in the non-acidified replicate using a Total Organic Analyzer (TOC-L Shimadzu) and ion chromatography (DIONEX ICS 3000) respectively. Additionally, the NH_4^+ concentration was determined using the modified Berthelot reaction (Krom, 1980). The ascorbic acid method 4500-P E (APHA, 2018) and the N-(1-naphthly) ethylenediamine dihydrochloride method 354.1 (USEPA, 1979) were used for PO_4^{3-} and NO_2^- determination respectively.

Surface sediments samples (< 2mm) were air-dried and ground with a pestle and mortar for homogenization. Their moisture content was determined in accordance with the American Public Health Association (APHA, 2018). One replicate of each sample was stored directly fresh at -20 ºC for DNA extraction and real-time quantitative polymerase chain reaction (qPCR) analysis.

Total carbon (TC) and nitrogen (TN) were analysed using a TruSpec CHNS Elemental Determinator (Leco Corporation). Total organic carbon (TOC) was determined as described in Method 2540 E (APHA, 2018). Total inorganic carbon (TIC) was calculated based on the

difference between TC and TOC. Inorganic nitrogen (NH_4^+ and NO_3^-) was measured after 1M KCl extraction (adapted from Mulvaney, 1996) using a Jasco V630 spectrophotometer. Total organic nitrogen (TON) was calculated by the difference between TN and inorganic nitrogen.

Based on the methodology described in a previous study (Unda-Calvo et al, 2017) to estimate the potential for metals to cause harmful non-carcinogenic effects amongst humans (children) to contaminated surface sediments, the hazard quotient (HQ) was calculated as shown in the following equation:

$$HQ = \frac{CDI \times B/100}{RfD} \quad (9.1)$$

Chemical daily intake (CDI) represents the possible entry of metals (mg kg^{-1} day^{-1}) into the human body; B is the percentage of bioaccessible metal pseudo-content in surface sediments when human gastric or intestinal environmental conditions were reproduced; and RfD (Reference Dose) is the estimated amount of daily oral exposure level for the population that is likely not to have an appreciable risk of deleterious effects during their lifetime. If the sum of HQ corresponding to the gastric and intestinal bioaccessibilities (∑HQ) for at least one of the metals (Cu, Zn, Cr, Ni and Pb) considered at each site exceeds 1, the surface sediment may be of concern for its potential harmful effects.

Analysis of biological variables. After DNA extraction from surface sediment samples (0.25 g of dry weight sediment) using a Power SoilTM DNA Isolation Kit, real-time qPCR was performed to measure denitrifying genes (*nirK, nirS* and *nosZ*), as described in Martínez-Santos et al. (2018). The nosZ:nir ratio, where nir is the sum of *nirK* and *nirS*, was calculated to represent the relative genetic capacity of denitrifying communities in surface sediments to reduce N_2O to N_2 with respect to the reduction of NO_2^- to NO.

Additionally, the activity of Nitrate Reductase (NR) —the enzyme responsible for reducing NO_3^- to NO_2^- in denitrification— for each surface sediment sample was analysed following the procedure established in a previous study (Unda-Calvo et al., 2019a). Given that NR activity was considered to be the main limiting step in denitrification, and in order

to estimate the capacity of the microbial communities harboured in the surface sediments for cumulative nitrate complete elimination, the authors decided to calculate the Response Time as shown in the following equation:

$$\text{Response Time (min)} = \frac{NO_3^- \text{ in sediment } (\mu g\ N\ g^{-1})}{NR\ (\mu g\ NO_2^- - N \text{ generated } g^{-1} min^{-1})} \quad (9.2)$$

All analytical procedures for both water and surface sediment samples were performed in triplicate.

9.3.3. Statistical analysis for river quality assessment

In Fig. 9.2 were summarized all the steps constituting the iterative process for the quality index development described below. The statistical techniques used for implementing these steps were also included: (i) Spearman correlation, one-way ANOVA (with Tukey's multiple test) and U-Mann Whitney non-parametric test for chemical variable selection; and (ii) Principal Component Analysis (PCA) for monitored data standardization, variables weighting and equation establishment. All statistical processing of the data described below were performed using SPSS 22.0 software.

9.3.3.1. Selection criteria for variables and reference conditions

In general, similar environmental variables were used in the quality indexes developed in different reports (Şener et al., 2017; Wang et al., 2017; Birch, 2018). However, since the aim of this research was to evaluate the influence of anthropogenic contamination on water and surface sediment quality, EC, DOC, nutrients (PO_4^{3-}, NO_3^-, NO_2^-, NH_4^+) and dissolved metals (Cu, Zn, Pb, Ni and Cr) were selected as indicators of water quality; in contrast, for the purposes of developing the sediment quality index, TOC, TIC, NO_3^-, NH_4^+, TON and the hazard quotient (∑HQ) of various metals (Cu, Zn, Pb, Ni and Cr) were used. All these variables are known to alter the biological quality of rivers, have a reduced analytical cost and help identify the impact of human activities (Martínez-Santos et al., 2018; Unda-Calvo et al., 2019a).

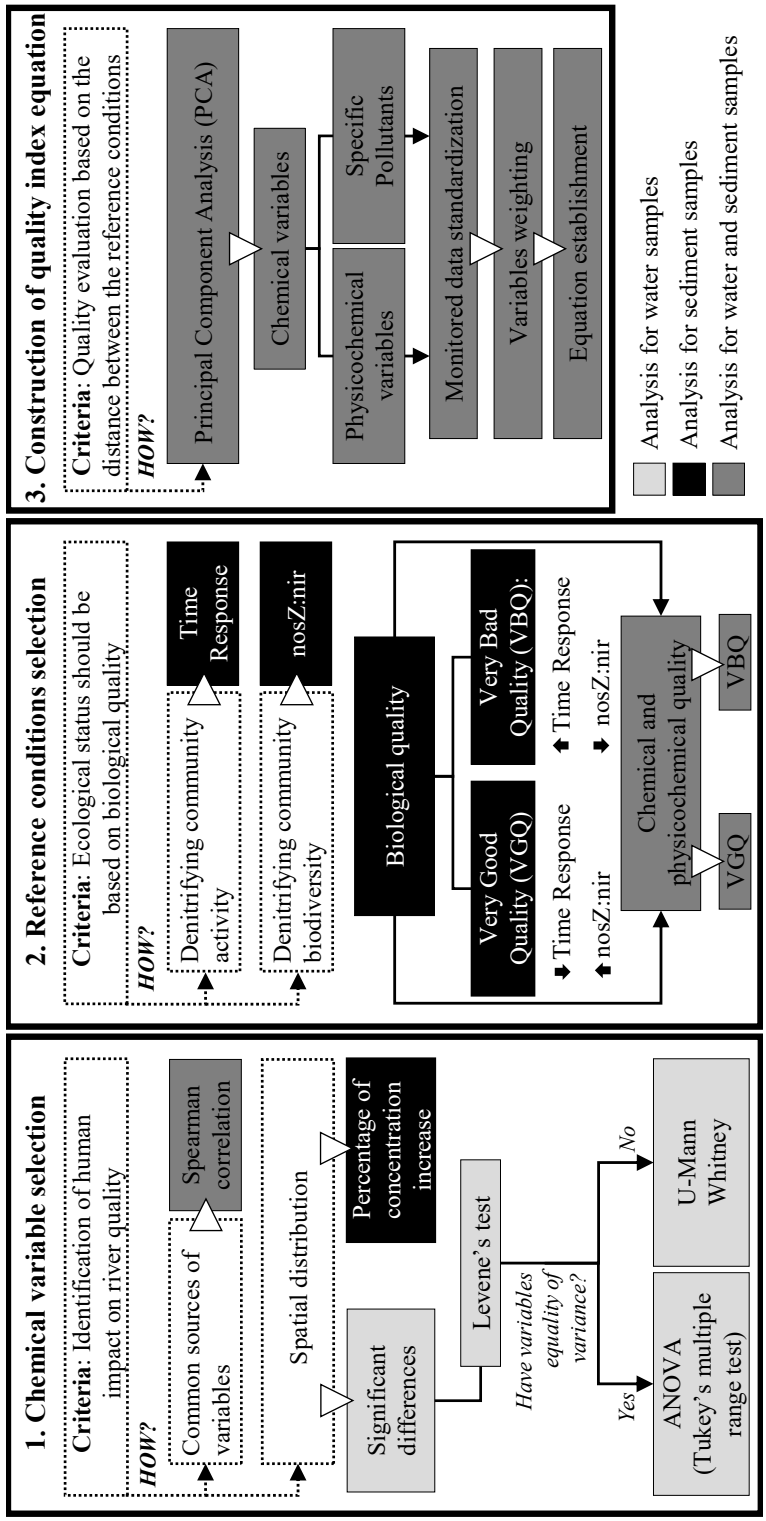


Fig. 9.2. *Flow diagram summarizing all the steps constituting the iterative process for the quality index development*

Firstly, all data were log-transformed in order to reduce skewness. After applying Levene's test to confirm which variables had equality of variance, we performed one-way ANOVA (taking $p < 0.05$ as significant, in accordance with Tukey's multiple range test) and the U-Mann Whitney non-parametric test respectively. These statistical analyses were performed with chemical variables of water samples from all sampling campaigns during the dry season, in an attempt to diminish the influence of the hydrological pattern on the human pressure detection. In addition to spatial distribution (percentage of concentration increase between locations up- and downstream of WWTPs effluents and untreated urban or industrial wastewater discharges), examination of significant differences among sampling sites helped us to recognize the anthropogenic origin of chemical variables. Also, a Spearman correlation analysis (non-parametric test) was performed with water and surface sediment chemical variables to identify common sources.

The establishment of reference conditions is an essential second step since the quality of any sampling location must be expressed as a deviation from those conditions. According to WFD Common Implementation Strategy guidance document No. 10 (REFCOND, 2003), reference conditions should be established based on the information provided by the relevant biological quality elements (i.e. composition and abundance of aquatic flora, composition and abundance of benthic invertebrate fauna or composition, abundance and age structure of fish fauna) defined for the classification of ecological status of a river. Alternatively, microorganisms are also particularly suitable for monitoring river quality due to their key role in biogeochemical cycling and their sensitivity and rapid response to any ecosystem perturbation (Chaer et al., 2009; Guo et al., 2012).

The microbial removal of nitrogen in aquatic ecosystems is of great interest for researches and managers since nitrate excess is linked to eutrophication, especially in coastal marine waters (Burgin and Hamilton, 2007). From the perspective of river water quality, complete denitrification still remains the most desirable nitrate removal pathway because it represents a permanent nitrogen sink (Arce et al., 2015) without depending on other N transformation processes or even S cycling (Burgin and Hamilton, 2007). In previous studies, two parameters related to the abundance and activity of denitrifying community in surface sediments were found particularly suitable for the monitoring of river quality: the nosZ:nir

ratio (Martínez-Santos et al., 2018) and the Response Time (Unda-Calvo et al., 2019a). These biological elements were indicative of the impact of treated and untreated effluents on the nitrogen cycling and reflected the ecological status of the Deba River catchment, where infringements for nitrate and ammonium, among other pollutants, were reported (URA, 2017). The nosZ:nir ratio represents the relative abundance of the nitrous oxide reductase (*nosZ*) gene —responsible for the conversion from N_2O to N_2— with respect to the nitrite reductase genes (nir = *nirS* + *nirK*) —responsible for the reduction of NO_2^- to NO in denitrification. Whereas *nirS* and *nirK* are considered to be universal to all denitrifiers (Azziz et al., 2017), not all were found to possess *nosZ* gene (Jones et al., 2008; Ligi et al., 2014). Thus, a low nosZ:nir ratio suggests that N_2O greenhouse gas emissions due to an incomplete denitrification may be expected. In addition, since the presence of denitrifying bacteria does not necessarily imply that they are operating (Veraart et al., 2017), we also used Response Time to ensure the capacity of the microbial community to completely eliminate the nitrate excess. For the purposes of establishing the reference conditions, we therefore used a combination of these parameters involved in microbial denitrification in surface sediments —the process by which NO_3^- and NO_2^- are reduced to NO and N_2O, and finally are converted to N_2 and returned to the atmosphere— as an indicator of river biological quality.

9.3.3.2. Multivariate data analysis and construction of quality index equations

For the purposes of developing both the water and sediment quality index, the variables were grouped into two categories: physicochemical variables and specific pollutants (Table 9.1). The calculation of the water and sediment quality indexes proposed in this work is an adaptation of the methodology established by the Basque Water Agency (URA, 2008) for developing a physicochemical quality index for water, applied to catchments in the Basque Country. After performing a PCA to the selected chemical variables, the mathematical procedure involved estimating the straight-line distance of each water or surface sediment sample to a hypothetical "Very Good-Bad Quality" (VGQ-VBQ) axis defined by the reference conditions on the factorial plane (Fig. 9.S1). In this way, we were able to quantify the degree of similarity (from 0 to 1) between the quality of the sampling and the reference sites, as required by the European Water Framework Directive (WFD, 2000/60/CE).

Firstly, the log-transformed monitored data ($M_{i,j}$) of the *jth* variable at each sampling site (i) —including reference conditions representing VGQ and VBQ— were standardized using their mean ($\bar{M}_j$) and standard deviation (σ_j) (Table 9.1) in order to give each chemical variable equal weight in the multivariate analysis. The statistical software returns the scores in PCA expressed as coordinates of the two-dimensional space defined by the selected principal components (eigenvectors associated with eigenvalues greater than or equal to 1 should be considered (Hooper, 2003) and subjected to an orthogonal varimax rotation to maximize the variance). However, the scores for each principal component (S_{pc}) could also be calculated from the standardized data and the corresponding loading factor (l_{pc}; Table 9.1) of the *jth* variable, as shown in Eq. (9.3).

$$S_{pc,i} = \sum_{j=1}^{n} \left(\frac{M_{i,j} - \bar{M}_j}{\sigma_j} x\, l_{pc,j} \right) \tag{9.3}$$

As shown in Fig. 9.S1 and Eq. (9.4), the quality index (QI) corresponds to the Euclidean distance from the orthogonal projection of the sampling site point on the VGQ-VBQ axis to the VGQ point (A), with respect to the Euclidean distance from the VBQ to VGQ point (B), the maximum distance. Consequently, the QI ranges from 0 (very poor quality) to 1 (excellent quality). Eqs. (9.5) and (9.6) were used to trigonometrically calculate Euclidean distances where, for B, p_1 and p_2 are the VGQ and VBQ points respectively; for C, the sampling site and the VGQ points respectively; and for D, the sampling site and the VBQ points respectively.

$$QI = 1 - \frac{A}{B} \tag{9.4}$$

$$A = \frac{C^2 - D^2 + B^2}{2B} \tag{9.5}$$

$$B^2, C^2, D^2 = \left(S_{pc1,p_1} - S_{pc1,p_2} \right)^2 + \left(S_{pc2,p_1} - S_{pc2,p_2} \right)^2 \tag{9.6}$$

Finally, by combining all these equations, we were able to express the QI for the water or surface sediment sample as a function of the monitored data ($M_{i,j}$) at each sampling site as follows:

$$\mathbf{QI} = \mathbf{K} - \sum_{j=1}^{n} \mathbf{w_j\ x\ M_{i,j}} \tag{9.7}$$

where K is a constant term and wj represents the weight attributed to each *jth* variable, both calculated according to Eqs. (9.8) - (9.11).

$$K = \frac{1}{2} + k_1 \text{ x } \sum_{j=1}^{n} \frac{\bar{M}_j \text{ x } l_{pc1,j}}{\sigma_j} + k_2 \text{ x } \sum_{j=1}^{n} \frac{\bar{M}_j \text{ x } l_{pc2,j}}{\sigma_j} + \frac{1}{2B^2} \text{ x } \sum_{pc1}^{pc2} \left(S^2_{pc,VBQ} - S^2_{pc,VGQ} \right) \quad (9.8)$$

$$w_j = k_1 \text{ x } \frac{l_{pc1,j}}{\sigma_j} + k_2 \text{ x } \frac{l_{pc2,j}}{\sigma_j} \quad (9.9)$$

$$k_1 = \frac{S_{pc1,VBQ} - S_{pc1,VGQ}}{B^2} \quad (9.10)$$

$$k_2 = \frac{S_{pc2,VBQ} - S_{pc2,VGQ}}{B^2} \quad (9.11)$$

In the absence of a QI classification standard, a value equal to 0.5 was established as a "good quality" threshold.

9.3.3.3. A new tool for assessing river quality susceptibility

A means for rapid recognition of the susceptibility of a sampling location due to the anomalous level of one or more chemical variables in water or surface sediment samples could be of interest for the decision-making process of basin managers. In this study, we proposed to calculate the Contribution Percentage (CP) of each variable (j) to QI at a sampling site (i), based on the weight (w_j) attributed to it in Eq. (9.7) and its log-transformed monitored data ($M_{i,j}$), as shown in Eq. (9.12). Note that the denominator refers to the QI value, without taking the constant term (K) into account.

$$CP_j\ (\%) = \frac{w_j x \frac{M_{i,j} - \bar{M}_j}{\sigma_j}}{\sum_{j=1}^{n} ABS \left| w_j x \frac{M_{i,j} - \bar{M}_j}{\sigma_j} \right|} \text{ x } 100 \quad (9.12)$$

While wj quantifies the influence (sign and magnitude) of the chemical variable on QI, $M_{i,j}$ data standardization makes it possible (i) to compare variables measured in different units; and (ii) to identify how far a sampling location deviated from the dominant trend in the catchment. Hence, as shown in Fig. 9.3, both should be considered when classifying the contribution of the chemical variable to the river water or surface sediment quality as positive or negative.

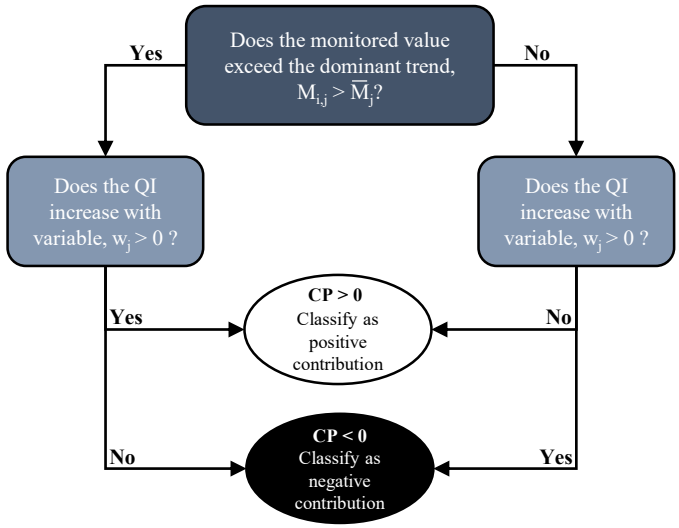


Fig. 9.3. *Flow diagram summarizing all steps involved in classifying the contribution of a chemical variable to water or surface sediment quality*

9.3.3.4. Analysis of pollutant exchange in the water-sediment interface

We evaluated *t*he effect of seasonality (two different hydrological conditions were established for dry and wet seasons) and thus of sediment dynamics (sedimentation or resuspension) on river quality by performing regression analysis between water and sediment QI.

9.4. Results and discussion

9.4.1. Environmental variables and reference conditions

In water samples from the dry season ($Q < 12$ m^3 s^{-1}), the highest median values of EC (746-1030 µS cm^{-1}, Table 9.S1) —which was strongly correlated ($\rho \geq 0.775$; $p < 0.01$) to the major ions (Cl^-, SO_4^{2-} , Na^+, Ca^{2+}, K^+ and Mg^{2+}; data not included)— were recorded in headwaters (D1, M1 and D2), indicating that groundwater circulation from rocks holding evaporites was being discharged into the river water (Fig. 9.1). However, increases to levels similar to those at D2 ($p > 0.05$; Table 9.S1) were also found downstream of the Mekolalde and the Apraitz WWTPs (D5 and D7 respectively), suggesting an additional anthropogenic source of river water EC. Indeed, the mean values in the effluents from these WWTPs (554 ± 101,

and 702 ± 120 µS cm^{-1} from Jan. 2015 to Jan. 2016 respectively), as provided by the Gipuzkoa Water Consortium (unpublished data), were higher than in the receiving waters from D4 and D6 respectively. In addition, DOC, all inorganic nitrogen forms (NO_3^-, NO_2^-, NH_4^+), as well as PO_4^{3-}, showed a strong positive correlation ($\rho \geq 0.386$; $p < 0.01$), supporting the idea of a common source for these chemicals. Despite the absence of significant differences throughout the catchment ($p > 0.05$; Table 9.S1), DOC rose between D2 and D3 (11%), D4 and D5 (4%), D6 and D7 (32%) and E1 and E2 (178%). Similarly, all nutrients (PO_4^{3-}, $NO3^-$, $NO2^-$ and NH_4^+) saw significant increases compared to headwaters ($p < 0.05$; Table 9.S1) at D3, D5, D7 and E2, which implies that WWTPs and, especially, untreated UWW are sources of organic carbon and nutrients. In the case of metals (Cu, Zn, Pb, Ni and Cr), the absence of significant differences among sites ($p > 0.05$; Table 9.S1) indicates the combined effect of different contamination sources (Fig. 9.1) throughout the catchment. As with organic contamination, the largest sources of metals at E2 are UWWs (significant differences ($p < 0.05$) between E1 and E2; Table 9.S1), except for Pb.

According to surface sediment samples (Table 9.S2), a significant correlation ($\rho = 0.905$; $p < 0.01$) between TOC and TON indicates that it was appropriate to consider the sum of NO_3^- and NH_4^+ as total inorganic nitrogen for the purposes of calculating TON. Apart from the deposition of vegetation at headwaters (D1 and E1), total organic carbon and nitrogen could be increased by WWTP effluents at D5 and, especially at D3. In addition, the high TIC measured at E2, D3 and D5 suggests that dissolved inorganic carbon from alkaline urban wastewaters and WWTPs effluents might have been precipitated onto surface sediments (Fig. 9.S3). As nutrients in the water, NO_3^- and NH_4^+ in surface sediment samples (Table 9.S2) registered the largest increases between D2 and D3 (145% and 372% respectively) and, especially, between E1 and E2 (319% and 1012% respectively). Finally, as reported in previous studies focusing on surface sediments from the Deba River, discharges of urban or industrial wastewaters, and even effluents from WWTPs throughout the entire catchment were all responsible for metals (Unda-Calvo et al., 2019b), whose availability made them the most hazardous for human health (Unda-Calvo et al., 2017). Although $\sum HQ > 1$ were not observed (Table 9.S2), the most potentially toxic location for children might be considered to be D2, D4, D5 and, above all E2 —where the highest bioaccessible Cu, Ni, Pb and Zn-Cr were measured respectively.

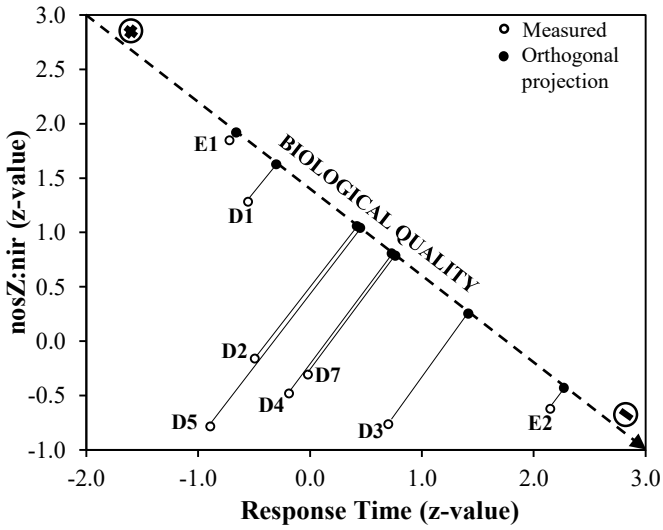


Fig. 9.4. *Relationship between two biological variables related to denitrifying community abundance (nosZ:nir) and activity (Response Time) for establishment of reference conditions*

As shown in Fig. 9.4, the biological quality of the river decreases with the nosZ:nir ratio and increasing Response Time, whereas it increases with the nosZ:nir ratio and decreasing Response Time. In this context, when the values measured from each sampling location are orthogonally projected onto the biological quality axis, the following order of decreasing quality was observed: E1 > D1 > D2 > D5 > D4 > D7 > D3 > E2. After identifying the sampling locations with the best and worst biological quality, physicochemical measurements registered at E1 and E2 were used as reference conditions: Very Good Quality (VGQ) and Very Bad Quality (VBQ) respectively. For the purposes of developing the water quality index, 90 and 10 percentiles of all sampling campaigns (N = 9) at E1 and E2 respectively were calculated and used as reference conditions (Table 9.S1). This statistical procedure was also used by the Basque Water Agency (URA, 2008) for setting the bandwidth of the chemical variables measured in water samples. The suitability of the reference conditions should be tested by the PCA, where the VGQ and VBQ must be the extreme points of the samples cloud in the two-dimensional space. As evidenced by Fig. 9.S1, the use of nosZ:nir ratio and the Response Time as biological quality indicators was appropriate for establishing the chemical reference conditions.

9.4.2. River quality assessment

As shown in Table 9.1, two principal components (PCs) with eigenvalues exceeding 1 were extracted, accounting for at least 68% of the total variance. Focusing on PCAs related to physicochemical variables, PC1 has positive correlation factors ($\rho > 0.50$) of DOC-PO_4^{3-}-NO_3^--NO_2^--NH_4^+ for water and of TIC-NO_3^--NH_4^+ for surface sediment. Conversely, PC2 has negative correlation factors ($\rho < -0.50$) of EC for water and TOC-TON for surface sediment. Considering that natural processes in the river have also been identified as major sources of EC, TOC and TON at headwaters (Table 9.S1 and Table 9.S2), PC2 represents a moderate anthropogenic influence while PC1 indicates a strong anthropogenic influence. Therefore, as the absolute standardized weights (w_j'; Table 9.2) indicate, physicochemical variables with largely anthropogenic origin had the highest influence on the water ($NO_2^- > PO_4^{3-} > NH_4^+ > NO_3^-$) and surface sediment ($NO_3^- > TIC > NH_4^+$) quality. Indeed, previous studies demonstrated that high-nutrient loads have a negative impact on denitrifying communities in river surface sediments, resulting in a greater Response Time (Unda-Calvo et al., 2019a) but a lower nosZ:nir ratio (Martínez-Santos et al., 2018). It should be noted that TOC and TON showed positive w_j (Table 9.2) since organic matter degradation provides a source of electrons for heterotrophic denitrification (Richardson, 2000), evidencing the capacity of the quality index development method to reveal any possible synergism or antagonism between the chemical variables.

When specific pollutants were taken into account, the principal components could be classified as representing a strong (PC1) or moderate (PC2) potential risk for the aquatic environment due to metal ecotoxicity (Table 9.1). Some metals (Cu, Ni or Zn) could be essential micronutrients for microorganisms but they become toxic at high concentrations (Alloway and Jackson, 1991; Zumft, 1997). In view of the negative sign of w_j (Table 9.2), the high metal contamination of the Deba River catchment had adverse effects on the denitrifying community, since it is more sensitive to dissolved Cr-Zn-Cu and to Zn-Pb-Cu ($\sum$HQ) in surface sediments. As observed, metal availability and, hence, metal bioaccessibility in sediments modulates their toxicity. Indeed, while Cr-Ni were predominantly retained in the mineral lattice of surface sediments, Pb was the most available metal (Unda-Calvo et al., 2019b).

Table 9.1. *Parameters for the calculation of quality index (QI): mean ($\bar{M}_j$), standard deviation (σ_j), reference conditions (Very Bad Quality (VBQ) and Very Good Quality (VGQ)) and loading factors (l_{pc}) from the PCA for the chemical variables measured in water and surface sediment samples from the Deba River catchment. Correlation factors, eigenvalues and explained variance for each Principal Component (PC) were also included*

		$\bar{M}_j \pm \sigma_j$	Reference conditions		Loading factors ($l_{pc,j}$)		Correlation factors	
			VBQ	VGQ	PC1	PC2	PC1	PC2
WATER								
Physicochemical Variables	EC	2.62 ±0.219	2.66	2.43	0.068	-0.924	0.127	-0.965
	DOC	0.584 ±0.160	0.819	0.213	0.360	0.136	0.673	0.142
	PO_4^{3-}	1.378 ±0.622	2.53	0.918	0.489	0.133	0.913	0.139
	NO_3^-	2.77 ±0.408	3.27	2.17	0.422	-0.246	0.789	-0.256
	NO_2^-	0.904 ±0.554	2.35	0.279	0.483	-0.087	0.902	-0.091
	NH_4^+	1.66 ±0.555	3.03	0.805	0.463	0.205	0.865	0.214
						Eigenvalues	3.49	1.09
						Explained variance	58%	18%
Specific Pollutants	Cu	0.319 ±0.263	0.462	-0.602	0.420	-0.277	0.641	-0.285
	Zn	0.837 ±0.595	2.37	-0.602	0.582	-0.011	0.889	-0.012
	Pb	-0.437 ±0.232	-0.129	-0.602	-0.186	-0.878	-0.284	-0.903
	Ni	0.197 ±0.469	0.350	-0.602	0.487	0.221	0.744	0.228
	Cr	-0.373 ±0.414	0.790	-0.602	0.462	-0.321	0.706	-0.329
						Eigenvalues	2.34	1.06
						Explained variance	47%	21%
SEDIMENT								
Physicochemical Variables	TOC	1.28 ±0.111	1.22	1.28	0.192	-0.657	0.303	-0.951
	TIC	1.18 ±0.313	1.61	0.702	0.480	0.340	0.757	0.493
	NO_3^-	0.933 ±0.250	1.41	0.789	0.535	0.254	0.844	0.369
	NH_4^+	0.897 ±0.369	1.56	0.512	0.617	-0.023	0.973	-0.033
	TON	0.239 ±0.233	0.143	0.214	0.256	-0.623	0.403	-0.903
						Eigenvalues	2.49	2.10
						Explained variance	50%	42%
Specific Pollutants	∑HQCu	0.260 ±0.250	0.0010	0.0040	-0.467	-0.379	-0.591	-0.554
	∑HQZn	0.422 ±0.463	1.04	-0.498	-0.660	0.069	-0.834	0.101
	∑HQPb	0.761 ±0.137	0.869	0.668	-0.580	0.315	-0.733	0.460
	∑HQNi	1.05 ±0.206	1.10	1.25	0.100	0.580	0.126	0.847
	∑HQCr	1.381 ±0.354	1.95	1.71	-0.010	0.645	-0.013	0.942
						Eigenvalues	1.60	2.13
						Explained variance	32%	43%

According to water QI spatial distribution (Fig. 9.5A), the highest median value was detected at headwaters (D1 and E1), and at the Mazmela (M1) and Oñati (O1) tributaries, while the lowest median value was at E2, both for physicochemical variables (I) and specific pollutants (II). Despite the high impact of industrial activities, there has been a particularly noticeable improvement in the water quality of the Oñati tributary to a level similar to those non-impacted sites (D1, M1 and E1), also registered by the Basque Water Agency (URA, 2018). However, the water quality was better when physicochemical variables were considered (all samples at O1 had a QI value above 0.5) than with specific pollutants (almost 25% of samples at O1 had QI value below 0.5).

Table 9.2. *Constant term (K) and weights (w_j) atrributed to selected chemical variables (j) for water and surface sediment QI equations construction (Eq. 9.7). Standardized weights were also calculated: $w_j' = w_j \times (\sigma_j / \sigma_{QI})$*

	WATER			SEDIMENT		
		w_j	w'_j		w_j	w'_j
Physicochemical Variables	K	1.733	[-]	K	1.535	[-]
	EC	-0.061	-0.054	TOC	0.219	0.075
	DOC	-0.296	-0.191	TIC	-0.389	-0.374
	PO_4^{3-}	-0.104	-0.261	NO_3^-	-0.497	-0.382
	NO_3^-	-0.141	-0.233	NH_4^+	-0.309	-0.350
	NO_2^-	-0.117	-0.262	TON	0.041	0.028
	NH_4^+	-0.109	-0.244			
Specific Pollutants	K	0.537	[-]	K	2.031	[-]
	Cu	-0.249	-0.350	∑HQCu	-0.468	-0.297
	Zn	-0.114	-0.363	∑HQZn	-0.446	-0.524
	Pb	-0.140	-0.174	∑HQPb	-1.449	-0.505
	Ni	-0.091	-0.228	∑HQNi	-0.044	-0.023
	Cr	-0.176	-0.390	∑HQCr	-0.133	-0.119

As regards the type of contamination source type (Fig. 9.1), effluents from WWTPs and, primarily, untreated UWW seem to have more negative effects on the physicochemical quality of the water than exclusively industrial wastewaters. Indeed, median water QI based on physicochemical variables was higher at D2-D4-D6 than at D3-D5-D7 and, especially, at E2 (Fig. 9.5A). In contrast to D2-D4-D6, more than 75% of samples had a QI value below 0.5 at D3-D5-D7 and at E2. Moreover, the percentage of samples with a QI value of below 0.5 increased from less than 25% at D2 to more than 25% at D4 and to more than 50% at D6, due to the indirect impact of the Mekolalde WWTP and the Ego tributary respectively.

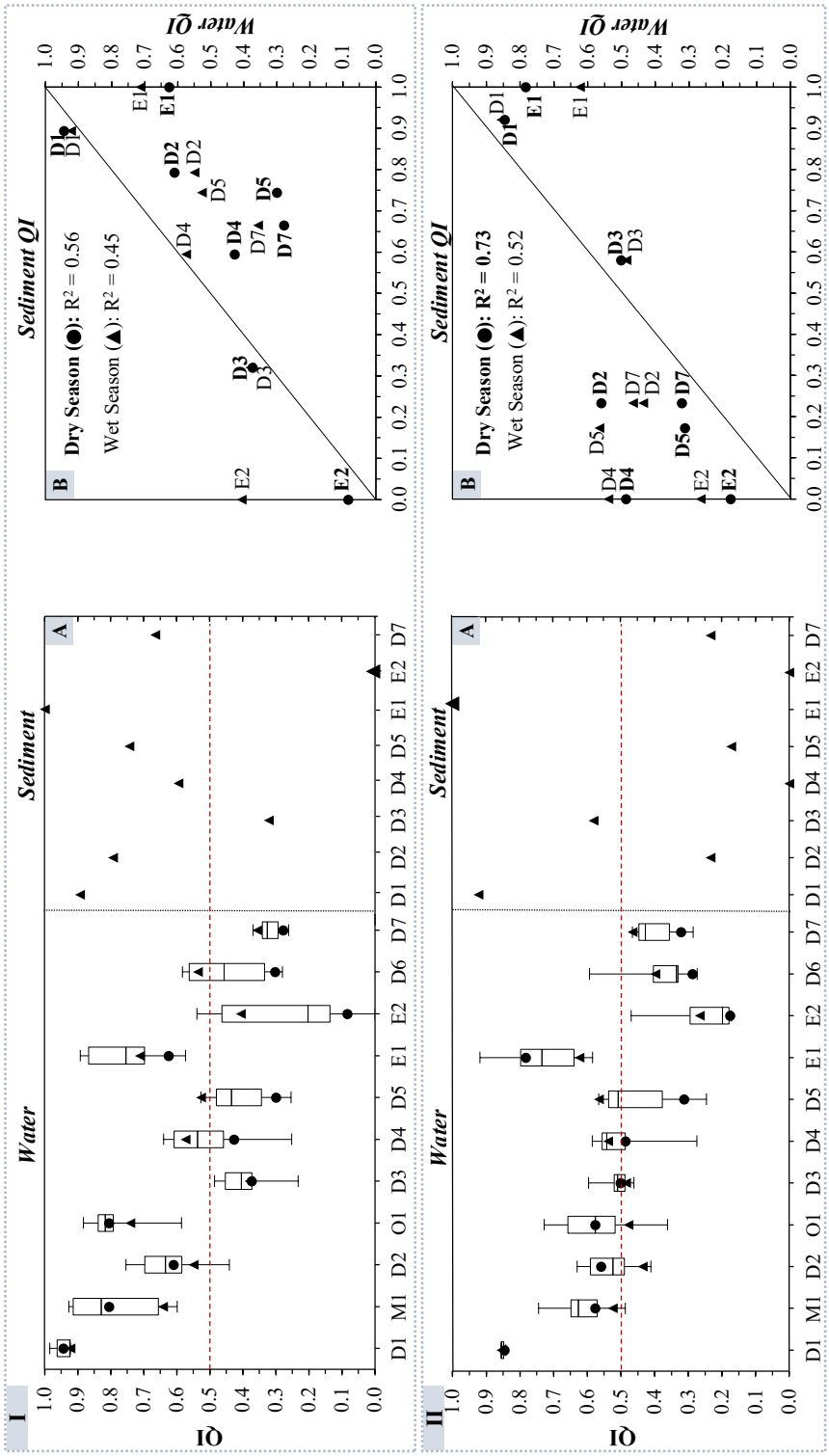


Fig. 9.5. *Spatial distribution of water and surface sediment QI throughout the catchment (A), and linear relationship between them (B) when physicochemical variables (I) or specific pollutants (II) are taken into account. For water, each box in (A) shows the 25th, 50th and 75th percentiles of values calculated for the research period Jan. 2015 — Jan. 2016. The 25th percentile of values calculated for the dry and wet seasons (circles and triangles respectively) are also plotted in A and used for linear relationships in B*

Conversely, it was not possible to distinguish the adverse effects of specific pollutants on water quality based on contamination source type. Compared to a 20% deviation in physicochemical QI median values, D2-D3-D4-D5 showed a higher homogeneity (almost 3% of deviation among median values) for specific pollutants, making the negative effects of industrial activities or WWTPs indiscernible.

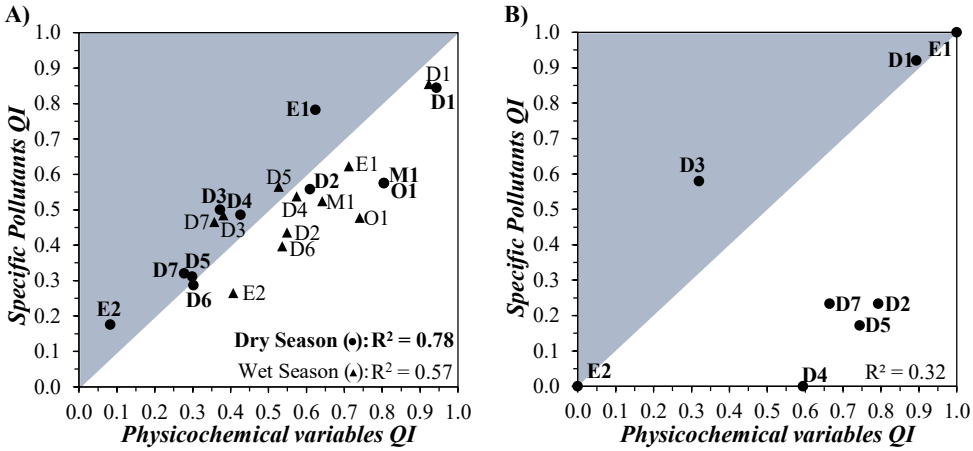


Fig. 9.6. *Linear relationships between QI related to physicochemical variables and specific pollutants in water samples during the dry and wet seasons (A), and in surface sediments (B). For water, the 25th percentile values from* Fig. 9.5B *were used. In the sampling sites within the grey area are, the physicochemical variables-dependent QI is worse than specific pollutants-dependent QI*

Surface sediment QI shows the same spatial trend as water (Fig. 9.5A), where the highest median values were found at headwaters (D1 and E1) while the lowest median values were found at E2, both for physicochemical variables (I) and specific pollutants (II). Moreover, the influence of anthropogenic activities on surface sediment quality was more pronounced. With regard to effluents from WWTPs, similar water quality was detected downstream of the Epele and Mekolalde WWTPs (D3 and D5 respectively), and further down, downstream of Apraitz WWTP (D7), due to the confluence of the Ego tributary. However, the increase in TIC, NO_3^- and NH_4^+ content in surface sediments downstream of Epele WWTP (Table 9.S2) was so considerable that the physicochemical quality was lower at D3 (QI < 0.5) and, even at D4, than at D7 (Fig. 9.5A). In addition, the load of specific pollutants from industrial activities led to extreme deterioration of surface sediment quality at D2 and D4 (75% and

100% of QI reduction compared to D1 and D3 respectively). The similarity between the spatial distribution of both QIs confirms the initial hypothesis that the combined analysis of water and surface sediment quality provides more comprehensive and detailed information on the susceptibility of the Deba River catchment. While a monthly determination of water QI could detect punctual stress situations, an annual evaluation of surface sediment QI provides advance information to early identify those human activities that, a priori, could compromise the river quality.

9.4.3. Effects of seasonality and sediment dynamics on water quality

We also evaluated the influence of hydrological conditions on the quality of the Deba River. Overall, the water quality was better from January to March than from May to November, as reflected by the 25th percentile of QI values calculated for the wet and the dry seasons respectively (Fig. 9.5A). However, this is not true at those sampling sites (D1, M1, D2, O1 and E1) where higher median values of the most influential environmental variables (PO_4^{3-}-NO_3^--NO_2^--NH_4^+) were observed for the wet season (Table 9.S1). Despite the fact that winter rainfalls increased river discharge, as measured at the Altzola gauging station ($R^2 = 0.65$; Fig. 9.1) and might therefore favour the dilution of contamination, previous findings suggested that the shallow depth of the water column together with the abrupt slope just upstream of these sites (Fig. 9.1) might promote a higher rate of sediment resuspension (Unda-Calvo et al., 2019b). Resuspension, together with changes in the pH or the oxidation conditions of the river environment caused by floods could promote the release of elements (e.g. organic matter, N, P or metals) trapped in surface sediments into the overlying water (Zhu et al., 2017; Camino Martín-Torre et al., 2017; Petranich et al., 2018), contributing to a decline in its quality. Indeed, a previous study (García-García et al., 2019) evaluating the variability of particulate metal pollution during flood events in the Deba River catchment, found that hydrodynamic processes were the main factor controlling the behaviour of particulate metals.

Sediment plays an important role in contamination transfer between aquatic environment compartments, acting as a sink or source for many pollutants. For a more accurate evaluation of the suitability of surface sediment analysis for rapid monitoring of water quality, linear regressions were applied between water and sediment QIs during two hydrological sets (Fig.

9.5B). As opposed to physicochemical variables (I), a flux of specific pollutants (II) in the sediment-water interface could be clearly identified, especially for the dry season. Metals are nonbiodegradable and, consequently, highly persistent. Physical and biochemical processes could therefore only control their mobility (Violante et al., 2010), thus facilitating our comprehension of metal exchange in the water-sediment interface. Conversely, organic matter and nutrients are subjected to a continuous transformation in river environments. Therefore, several processes should also be considered for a realistic modelling of organic matter and nutrient fluxes between water and sediment (Thouvenot et al., 2007). This illustrates how an annual determination of bioaccessible metal in surface sediments can be applied as a suitable strategy for prospective monitoring metal-dependent water quality, especially during low flow periods, when the river quality might be severely compromised.

Once again, these findings demonstrate that sediment QI also provides supplementary information related to specific pollutants since water QI was not able to distinguish the impact of different metal contamination sources, as discussed above. However, it should be noted that surface sediments underestimate water quality based on specific pollutants (II), except at D1, D3 and E1 (Fig. 9.5B). The high capacity of surface sediments to adsorb metals from the overlying water has been extensively discussed (Li et al., 2014; Lundy et al., 2017; Chu et al., 2019), and organic matter content has been reported as a key factor controlling metal adsorption (Yang et al., 2010). Indeed, Zn, Cu and Cr adsorption onto surface sediments, which were notably bound to the oxidizable fraction (from 17.7% to 30.3% of the pseudo-total content; Unda-Calvo et al., 2019b), seems to be favoured by a positive organic matter gradient between water and sediments (Fig. 9.S2).

The good linear relationship observed between the QI that is dependent on physicochemical variables and the QI that is dependent on specific pollutants in water samples taken during the dry season (Fig. 9.6A) suggests a common source of both categories of chemical variables throughout the catchment, with physicochemical variables posing a higher threat to the water quality downstream of D2 and in the Ego tributary. Conversely, during the wet season, the pattern of behaviour was more closely related to surface sediments, with specific pollutants greatly conditioning their quality (Fig. 9.6B). The alteration in metal performance with hydrological conditions in the river shows that surface sediments act as a

sink of dissolved metals during sedimentation in the dry season, whereas in the wet season there is potential for mobilization of available metals in surface sediments to the overlying water during sediment resuspension.

In order to identify the key chemical variable responsible for the decline in water and surface sediment quality throughout the river during the dry season, we calculated the relative contribution of each variable (in percentage) (Fig. 9.7). Sampling sites with water QI (25th percentile of values) above 0.5 for the dry season (D1, M1, D2, O1 and E1; Fig. 9.5A) showed positive contributions of almost all physicochemical variables, except for EC (specially at D2) and DOC (mainly at E1). While groundwater circulation from evaporitic rocks was identified as being responsible for high EC values from D1-M1 to D2, soil erosion and the subsequent solubilization of organic matter could cause high DOC contents in water at E1. On the other hand, sampling sites with water QI (25th percentile of values) below 0.5 for the dry season (D3, D4, D5, E2, D6 and D7; Fig. 9.5A) showed negative contributions of all physicochemical variables, with DOC and inorganic nitrogen (NO_3^-, NO_2^- or NH_4^+) being the key factors in declining water quality. Each type of contamination source (Fig. 9.1) appears to alter the nitrogen cycle differently along the length of the river. While at sites immediately downstream of WWTPs (D3, D5 and D7), NO_3^- content in water is of greatest concern, the water quality at E2 was deteriorated by NH_4^+ and, primarily, NO_2^- from untreated UWWs. Sites with direct impact of industrial activities but indirect influence of effluents from WWTPs (D4) or UWWs (D6) showed a combination of high NO_3^-+NO_2^- negative contribution to water quality (both between 20-30%; Fig. 9.7). With regard to specific pollutants, all sampling sites except D1 showed the negative contribution of some metal. Despite being unpolluted, headwaters from the Mazmela (M1) and the Ego (E1) tributaries presented a negative CP of Pb to water quality, possibly as a result of the solubilization of mobile Pb in surface sediments. Among the sites with a water QI (25th percentile of values) of below 0.5 for the dry season (D4, D5, E2, D6 and D7; Fig. 9.5A), those which showed high negative CP of Cr+Cu+Zn (at least 50%; Fig. 9.7) had the worst water quality.

Only surface sediments from D3 and E2 showed physicochemical QI values below 0.5 (Fig. 9.4A), with NO_3^- being the main physicochemical variable contributing to the decline of sediment quality. The high Response Time and the low nosZ:nir ratio measured at these

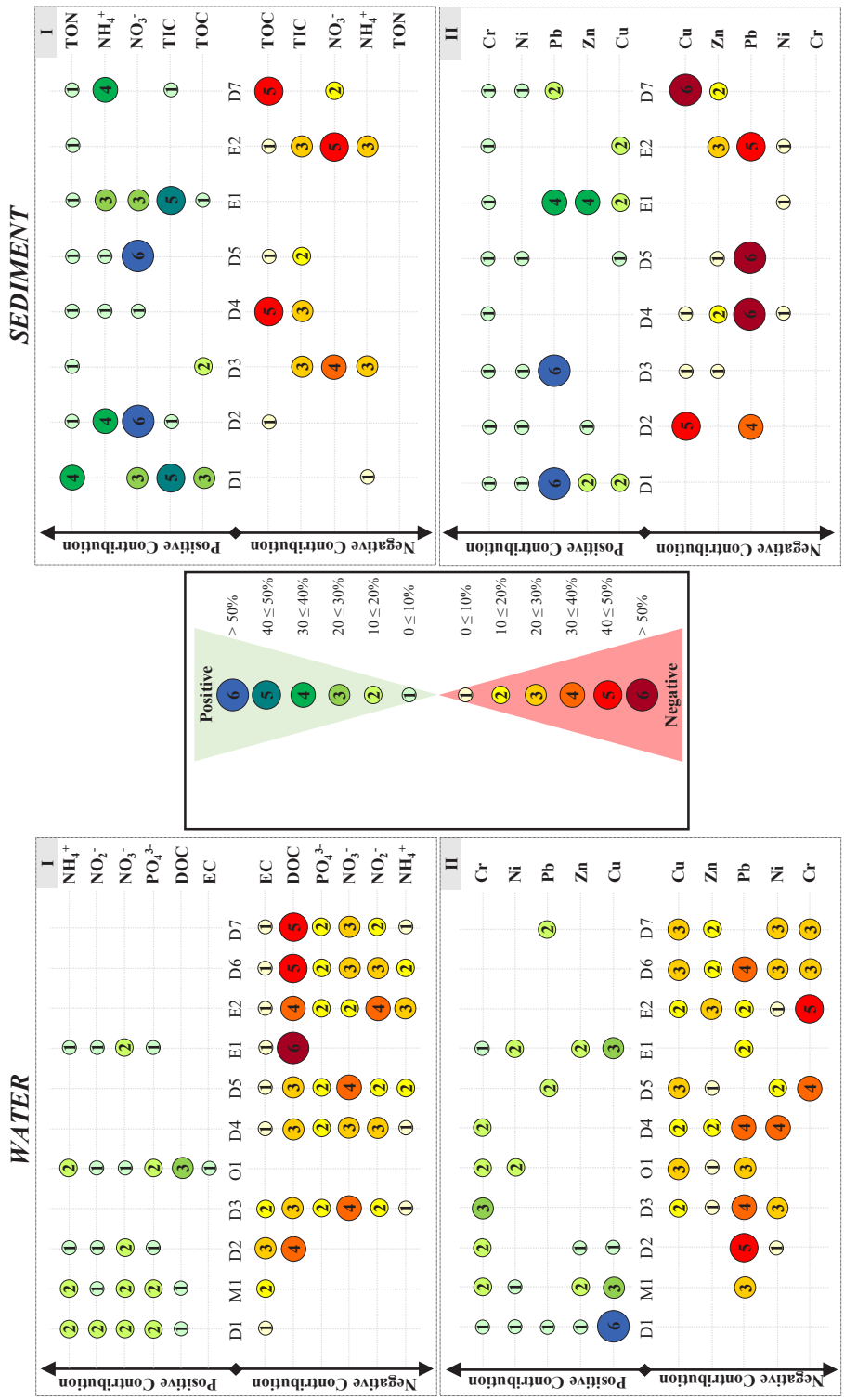


Fig. 9.7. *Spatial distribution of chemical variables (I: physicochemical variables; II: specific pollutants) contribution to water and surface sediment quality. For water, the 25th percentile of CP calculated for the dry season sampling campaigns was considered*

sites (Table 9.S2) indicate a low potential for denitrifying the excess of nitrates. On the other hand, surface sediments from D2, D4, D5, E2 and D7 presented a low quality (QI < 0.5; Fig. 9.4A), mainly due to the high negative CP for Cu, Zn and, principally, Pb (>50% of negative contribution at D4 and D5).

Fig. 9.8 summarizes all the steps involved in the development of a new multimetric index, and its contribution to the river quality evaluation. Specifically, water and sediment QIs helped us not only to identify anthropogenic sources of pollution, but also to study the influence of hydrological regime seasonality on water quality and sediment dynamic. In fact, a cyclical behaviour of surface sediments was observed, since they act as sink for pollutants instead of source during the low flow season. Furthermore, in conjunction with the QI, calculating the CP of chemical variables to river quality is also an essential tool for basin managers wanting to specifically evaluate the decline in quality at each sampling site. As an example, despite the low influence (w_j; Table 9.2) of DOC and dissolved Pb on water quality compared to other chemical variables used to calculate the QI, they were the main cause of the bad quality registered at D6 and D7 (Fig. 9.7).

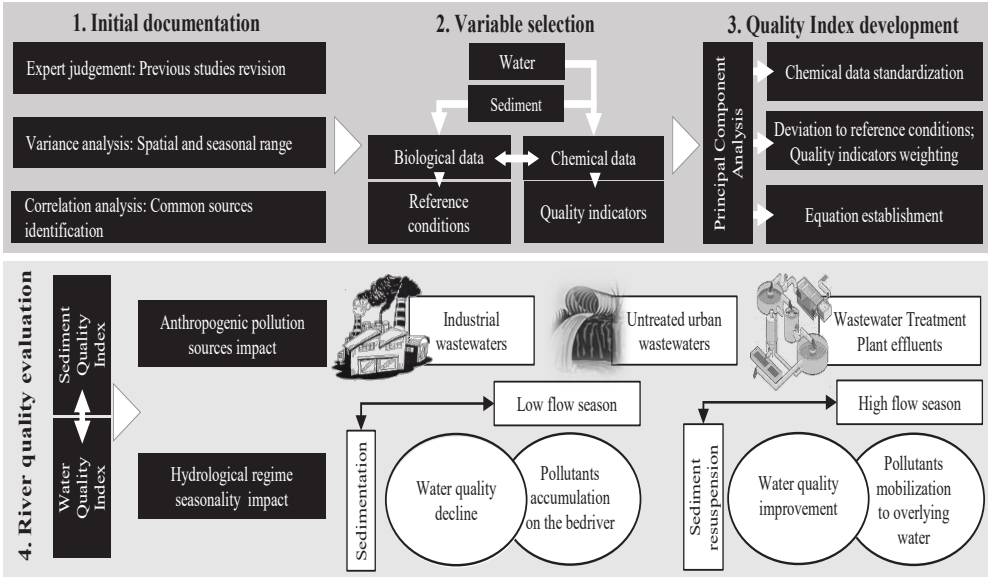


Fig. 9.8. *Schematic summary including all the steps involved in the quality index development for water and sediment, and its contribution to the river quality evaluation*

9.5. Conclusions

The mission of managers is to achieve sustainable development of an urban ecosystem and meet together the environmental targets set out in the European Water Framework Directive (WFD, 2000/60/EC). In this task, recognition of the susceptibility of a catchment subjected to multiple human pressures is essential for effective decision-making. The application of multivariate statistical analysis increased the level of objectivity into the quality index development. Techniques such as Spearman correlation, one-way ANOVA or U-Mann Whitney non-parametric test helped us to select with reliability the most appropriate chemical variables for the evaluation of human impact on river quality. In addition, performing a PCA allowed us (i) to assign a weight or relative influence on overall quality to each chemical indicator, based on the statistical distribution (mean and standard deviation) of all monitored data; (ii) to consider any possible synergic/antagonist effect; and (ii) to express the quality as a deviation from reference conditions. In this context, the implementation of two parameters related to the abundance (nosZ:nir ratio) and activity (Response Time) of denitrifying bacteria in surface sediments as biological quality indicators provided suitable information for the establishment of reference conditions, thus complying with the WFD (2000/60/CE) requirements.

The multimetric index proposed in this study has proved to be useful tool to assist water authorities in the identification of anthropogenic contamination sources that could compromise the river quality especially during certain months of the year. As observed, the discharge of industrial, WWTP and, mainly, untreated UWW effluents into the Deba River catchment increased organic matter, nutrient and metal content in water and surface sediments, thus contributing to a lower QI, especially in the Ego tributary, and mid- and downstream area of the main river. Conversely, high flows helped to dilute contamination and, consequently, a higher water QI was observed from January to March. Hydrological conditions also appeared to cause a different behavioural pattern in sediments, acting as a metal sink in the dry season, whereas in the wet season, there is potential for mobilization of metals to water during sediment resuspension. Therefore, an annual determination of surface sediments QI is suitable way of prospective monitoring water quality, especially during low flow periods, identifying sites that might merit particular attention and planning future

strategies for river quality improvement. Two limitations were found: (1) surface sediment was not appropriate for early monitoring the physicochemical quality of the water due to the continuous transformation of organic matter and nutrients; and (2) a multimetric index did not provide concise and definitive quality information. It is therefore proposed to calculate both the QI and the CP of chemical variables in order to specifically evaluate the water and surface sediment quality by identifying pollutant/s of concern at each location.

9.6. References

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9.7. Supplementary material

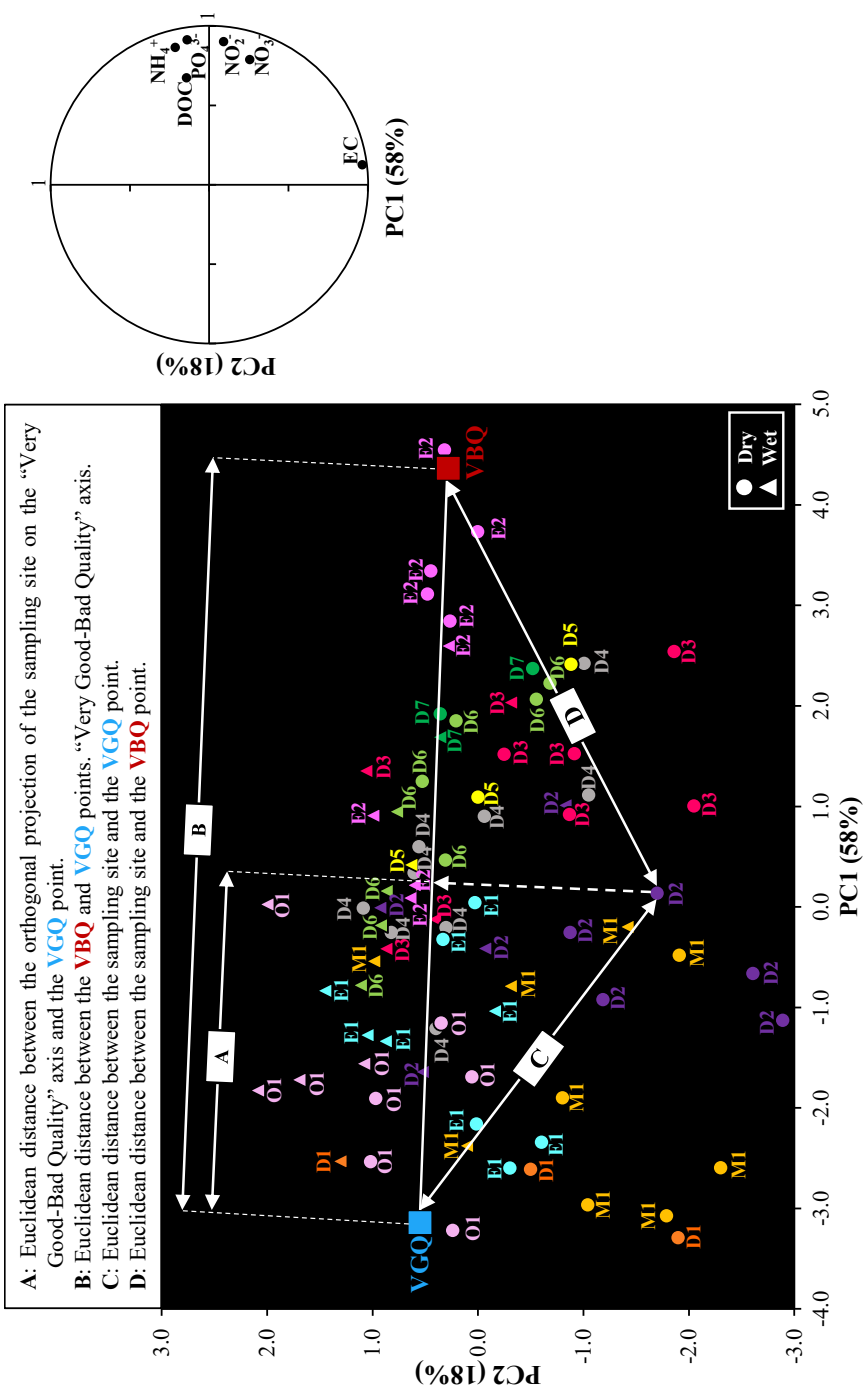


Fig. 9.S1. *As an example of the mathematical procedure, the Principal Component Analysis applied to the physicochemical variables of water samples for all sampling sites and for both seasons (dry: circles; wet: triangles)*

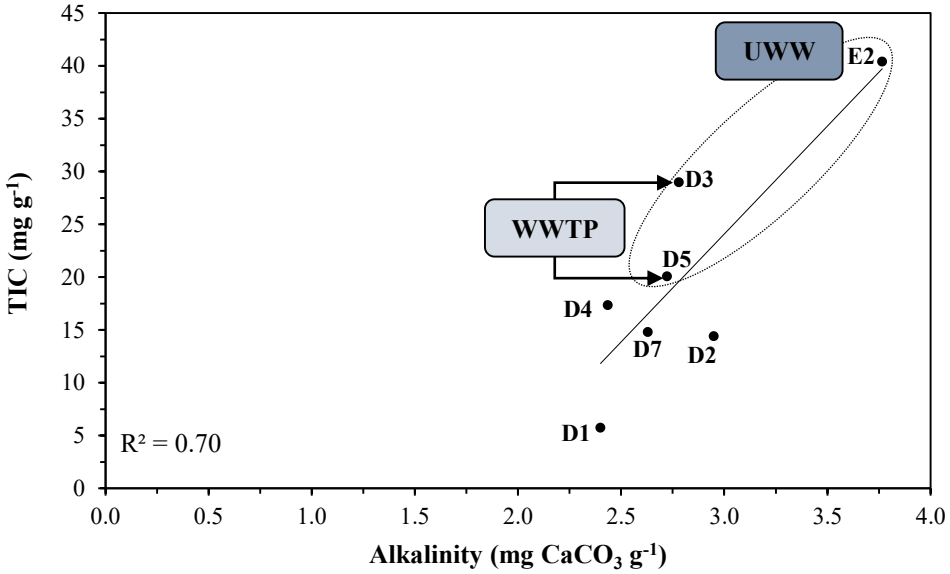
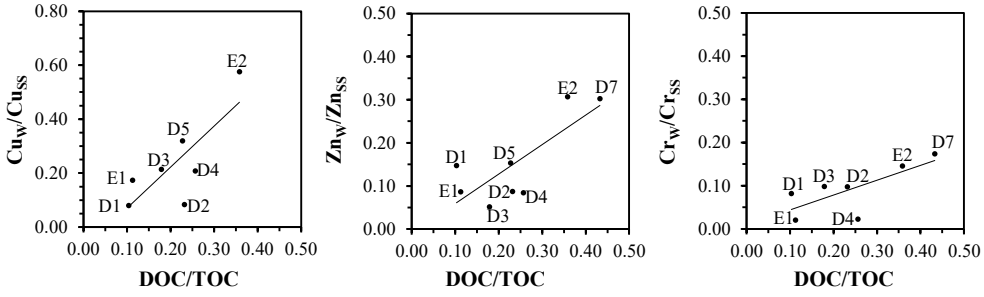


Fig. 9.S2. *Linear relationships between organic carbon and metal water-to-sediment gradients for Cu, Zn and Cr during the dry season. Median values obtained for the dry season for DOC and dissolved metal in water from* Table 9.S1*, and TOC and bioaccessible metal content in surface sediment (µg g-1) for the calculation of HQ from* Table 9.S2 *were used*



$[Cu_W/Cu_{SS}]$= 1.51[DOC/TOC] - 0.08 R^2 = 0.61 $[Zn_W/Zn_{SS}]$= 0.69 [DOC/TOC] - 0.01 R^2 = 0.61 $[Cr_W/Cr_{SS}]$= 0.34 [DOC/TOC] - 0.009 R^2 = 0.54

Fig. 9.S3. *Linear relationship between total inorganic carbon (TIC) content in surface sediments and water alkalinity (median values obtained for the dry season; data not included)*

Table 9.S1. *Median values of chemical variables measured in water samples of all sampling sites during the dry ($Q < 12\ m^3\ s^{-1}$) and wet ($Q > 12\ m^3\ s^{-1}$) seasons, and water quality reference conditions (Very Good Quality (VGQ) and Very Bad Quality (VBQ)). When measured values for each sampling campaign (N) were below the detection limit (d.l.), the 50% of the d.l. was considered. For the dry season, maximum values are shown in bold and different uppercase letters within the same parameter indicate that medians are significantly different at p = 0.05 among sampling sites. Once all data were log-transformed in order to reduce the skewness and the Levene's test confirmed which parameters had equality of variance (*) or not (**), one-way ANOVA (taking $p < 0.05$ as significant, in accordance with Tukey's multiple range test) and the U-Mann Whitney non-parametric test were performed, respectively, to analyse the differences*

		EC**	**DOC****	**PO_4^{3-}***	**NO_3^-***	**NO_2^-***	**NH_4^+****	**Cu****	**Zn***	**Pb****	**Ni****	**Cr****
Season	**Site**	µS cm^{-1}	mg L^{-1}	µg P L^{-1}	µg N L^{-1}	µg N L^{-1}	µg N L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}	µg L^{-1}
Dry	D1	746^{abcd}	2.85^{ab}	1.27^{a}	233^{ab}	1.57^{a}	10.4^{ab}	0.250^{ab}	4.11^{abc}	0.250^{a}	0.475^{abcd}	0.250^{abc}
(N = 5)	M1	956^{bc}	3.47^{ab}	4.90^{a}	243^{b}	2.38^{a}	11.0^{b}	0.920^{b}	1.67^{bc}	0.650^{a}	0.760^{ac}	0.250^{b}
	D2	1030^{c}	4.26^{ab}	10.3^{ab}	328^{abc}	6.22^{ab}	36.4^{abc}	1.11^{bc}	4.90^{abc}	0.730^{a}	1.64^{ab}	0.250^{b}
	D3	652^{bc}	4.73^{ab}	103^{c}	1918^{e}	12.2^{bc}	47.5^{acd}	1.48^{ac}	5.89^{bd}	0.580^{a}	3.25^{bd}	0.250^{b}
	O1	267^{d}	2.20^{b}	5.48^{a}	301^{abd}	2.86^{a}	11.6^{b}	1.26^{abc}	5.70^{abc}	0.660^{a}	0.690^{c}	0.250^{b}
	D4	393^{ab}	4.07^{ab}	38.9^{bcd}	1585^{aef}	12.3^{bc}	62.0^{ce}	1.41^{ac}	9.78^{bd}	0.250^{a}	2.30^{abd}	0.250^{bc}
	D5	511^{abcd}	4.22^{ab}	110^{bcd}	2116^{cde}	26.6^{bcd}	93.6^{ce}	1.65^{abc}	12.1^{bde}	0.250^{a}	7.28^{abcd}	2.45^{abc}
	E1	422^{a}	2.13^{ab}	10.5^{ad}	229^{bf}	3.59^{ab}	20.0^{ab}	0.550^{b}	0.650^{c}	0.780^{a}	0.250^{c}	0.250^{b}
	E2	420^{a}	5.92^{a}	182^{c}	1606^{de}	99.5^{d}	664^{f}	1.81^{ac}	78.8^{e}	0.610^{a}	1.55^{ab}	3.06^{a}
	D6	370^{a}	4.19^{ab}	56.6^{bcd}	1238^{ae}	21.4^{cd}	140^{de}	1.86^{a}	18.4^{ade}	0.250^{a}	6.33^{d}	0.900^{c}
	D7	441^{abcd}	5.55^{ab}	119^{bcd}	2177^{cde}	27.3^{bcd}	96.9^{cd}	1.71^{abc}	22.5^{abe}	0.250^{a}	6.95^{abcd}	0.955^{abc}
Wet	D1	190	3.07	2.64	226	1.82	12.8	0.250	1.80	0.250	0.820	0.250
(N = 4)	M1	407	3.92	9.72	468	3.48	27.3	1.59	3.60	0.250	2.53	0.250
	D2	396	4.93	24.9	453	4.75	38.6	2.18	7.51	0.250	2.17	0.250
	D3	359	4.89	53.4	456	9.37	78.7	2.12	4.86	0.250	2.61	0.250
	O1	184	3.65	13.4	292	2.77	27.6	2.13	6.59	0.250	1.06	0.250
	D4	276	3.35	25.8	533	6.30	46.2	1.61	6.18	0.250	1.70	0.250
	D5	279	4.71	34.3	1027	8.95	31.2	1.63	24.3	0.250	3.60	0.250
	E1	301	4.72	19.3	265	2.65	20.2	1.53	3.57	0.250	0.585	0.250
	E2	312	3.97	45.1	634	13.8	140	2.65	8.60	0.250	1.35	1.29
	D6	271	3.96	39.1	545	8.20	46.4	2.02	9.68	0.250	2.34	0.520
	D7	365	4.50	97.7	1043	27.5	120	1.63	24.3	0.250	3.60	0.250
	VGQ	268	1.63	8.27	149	1.90	6.38	0.250	0.250	0.250	0.250	0.250
	VBQ	454	6.58	342	1853	223	1079	2.9	232	0.743	2.24	6.22
	d.l.	0.01	0.004	2.5	20	2	5	0.5	0.5	0.5	0.5	0.5

Table 9.S2. *Environmental variables determined in the surface sediments at each sampling site in October 2015*

	TOC	TIC	NO_3^-	NH_4^+	TON	∑HQ (x10^3)					Response Time	nosZ:nir (x10^7)
						Cu	Zn	Pb	Ni	Cr		
Site	mg g^{-1}	mg g^{-1}	µg N g^{-1}	µg N g^{-1}	mg g^{-1}	[-]	[-]	[-]	[-]	[-]	min	[-]
D1	27.5	5.75	6.02	8.96	3.19	0.985	1.18	4.12	8.76	12.9	23.0	5.62
D2	18.4	14.4	5.97	4.26	1.89	4.19	2.37	6.37	6.58	10.9	24.2	3.10
D3	26.4	29.0	14.6	20.1	3.97	2.26	4.72	3.58	9.67	11.0	47.0	2.05
D4	15.9	17.3	8.39	7.31	1.28	2.16	4.91	8.09	27.9	46.5	30.0	2.54
D5	18.5	20.1	4.41	6.42	1.49	1.65	3.36	8.25	8.14	14.3	16.5	2.01
E1*	18.9	5.04	6.15	3.25	1.64	1.01	0.317	4.65	17.9	51.8	19.9	6.61
E2*	16.5	40.4	25.8	36.1	1.39	1.00	10.9	7.40	12.6	89.5	74.7	2.29
D7	12.8	14.8	9.38	3.57	0.787	3.60	3.16	5.65	9.08	23.4	33.3	2.85

(*) Sediment quality reference conditions: E1 (Very Good Quality (VGQ)) and E2 (Very Bad Quality (VBQ)).

10

Conclusiones y recomendaciones

"In order to seek truth, it is necessary once in the course of our life to doubt, as far as possible, of all things"

René Descartes (1596-1650)

10. Conclusiones y recomendaciones

Se ha considerado conveniente que cada uno de los capítulos de esta Tesis Doctoral presente, de manera individual, un apartado detallado de las conclusiones específicas sobre los diferentes objetivos definidos para el desarrollo de cada uno de ellos. Por tanto, a continuación, se exponen de manera abreviada los principales resultados extraídos del conjunto de este trabajo para la consecución del objetivo general. Asimismo, se realizan una serie de recomendaciones para su aplicación y/o el desarrollo de futuras líneas de investigación que se derivan del presente estudio.

10.1. Conclusiones

En el entorno de la Gestión Integrada de Recursos Hídricos (GIRH), la función de los gestores consiste, primordialmente, en la consecución de un desarrollo sostenible de los ecosistemas urbanos en paralelo con el cumplimiento de los objetivos ambientales establecidos en la Directiva Marco del Agua (DMA, Directiva 2000/60/CE), labor en la que el reconocimiento de la susceptibilidad de dicho ecosistema se convierte en una herramienta esencial para una toma de decisiones efectiva. En esta coyuntura, la realización de esta Tesis Doctoral ha permitido remarcar el papel fundamental de los sedimentos en la dinámica de

contaminantes en los sistemas acuáticos y, por ende, en la evaluación del estado ecológico de una cuenca hidrográfica, lo cual ha sido de gran utilidad para valorar los cambios en la gestión de vertidos de aguas residuales urbano-industriales acometidos, en los últimos años, en la cuenca del río Deba (Gipuzkoa, País Vasco).

La consideración del Sedimento como elemento integrador del medio acuático (Agua-Sedimento-Biota) y su importante función como fuente, sumidero y vector de transporte de contaminantes han servido como piedra angular para la evaluación del estado ecológico de la cuenca del río Deba para, como último fin, sugerir una estrategia complementaria a la establecida en la normativa europea y estatal, centrada fundamentalmente en el control de la calidad biológica, química y fisicoquímica de la matriz agua.

De hecho, una conclusión importante de esta investigación es la necesidad de incluir el Sedimento en los programas de monitoreo para el control del estado ecológico de una cuenca hidrográfica. Concretamente, el desarrollo y aplicación de un índice multimétrico a partir de indicadores de calidad química y fisicoquímica, así como biológica, ha evidenciado que, al menos, un muestreo y análisis anual del sedimento aporta una valiosa información prospectiva sobre el estado y evolución de la calidad de las aguas en los ríos, especialmente, en lo relativo a metales, identificando aquellas zonas susceptibles de polución y posibilitando la definición de nuevas estrategias futuras de sostenibilidad.

En este contexto, la elección de los indicadores de calidad del sedimento capaces de reflejar la influencia de la actividad humana sobre el estado ecológico de la cuenca ha resultado ser una etapa decisiva. A pesar de que la DMA (2000/60/CE) no incluye la comunidad bacteriana como indicador biológico para la evaluación del estado ecológico de los ríos, el estudio de su abundancia, biodiversidad (estructural y funcional) y actividad metabólica en los sedimentos, con especial incidencia en las bacterias desnitrificantes, ha demostrado ser una herramienta adecuada para predecir cambios en las funciones ecosistémicas de los ríos y analizar su vulnerabilidad frente a los vertidos de aguas residuales tratadas y no tratadas. Se ha observado que estos no solo alteran la distribución espacial y composición de las comunidades bacterianas involucradas en los ciclos biogeoquímicos del nitrógeno y el azufre —lo que conduce a una posible emisión de gases de efecto invernadero (N_2O) como producto de una desnitrificación incompleta—, sino también exceden su capacidad metabólica para eliminar

completamente el exceso de nitratos y garantizar la sostenibilidad de la cuenca.

La implantación de medidas correctoras en la cuenca del río Deba —entre otras, la puesta en funcionamiento de cuatro estaciones depuradoras de aguas residuales (la última en 2012) o la construcción del colector Ermua-Eibar y su posterior conexión a la depuradora de Apraitz en junio de 2014—, impulsaron una considerable mejora del estado biológico y fisicoquímico de algunos tramos del río. Sin embargo, junto con tales progresos, la Agencia Vasca del Agua–Ur Agentzia constató en su último informe (URA, 2019) el grave incumplimiento de los objetivos medioambientales en algunas masas de agua, habida cuenta del mal potencial ecológico del afluente Ego, entre otras.

En este trabajo, ciertamente, el control de la contaminación (de enero del 2015 a enero del 2016) a lo largo del cauce principal y los afluentes de la cuenca del río Deba ha puesto de manifiesto que los efluentes de las estaciones depuradoras y, muy especialmente, los vertidos de aguas residuales urbano-industriales sin tratar aumentan el contenido en materia orgánica, nutrientes y metales, entre otros, tanto en la matriz agua como en el sedimento. Posteriormente, la evaluación espacial conjunta de la calidad química, fisicoquímica y biológica del Sedimento (campaña de muestreo de octubre del 2015) ha permitido corroborar el mal estado ecológico actual de algunas masas de agua; concretamente, el tramo medio-bajo del cauce principal y, primordialmente, el afluente Ego.

De los resultados de esta investigación se desprende, por tanto, el gran potencial bioindicador de la comunidad bacteriana presente en los sedimentos, cuya rápida respuesta ante cualquier perturbación en el ecosistema es capaz de pronosticar, a diferencia de la matriz Agua, el impacto de distintas fuentes de contaminación sobre el estado ecológico de los ríos. De esta manera, ayuda a valorar, con inmediatez, la idoneidad de las medidas relacionadas con el saneamiento y depuración de las masas de agua, fase imprescindible para futuras actuaciones. Además, la complejidad y diversidad de procesos que tienen lugar en la interfase Agua-Sedimento hacen, en el caso de la materia orgánica y los nutrientes, sumamente difícil prever la calidad fisicoquímica del agua a partir del análisis del sedimento. Sin embargo, la medición de la actividad enzimática de la comunidad bacteriana, involucrada en la transformación de estos elementos, es también un parámetro útil para vaticinar su evolución, ya que constituyen la fuente o producto de su metabolismo.

Por otro lado, la determinación conjunta del contenido total, especiación química y bioaccesibilidad de los metales como indicadores químicos de la potencialidad tóxica de los sedimentos ha permitido detectar las fuentes antropogénicas de estas especies. Simultáneamente, se ha evaluado el riesgo que suponen tanto para la salud ecosistémica como humana con mayor exactitud y de forma más realista que las actuales directivas, que establecen las normas de calidad ambiental (NCA) en función, únicamente, de su contenido total. Este trabajo ha demostrado que la bioaccesibilidad y, por tanto, la toxicidad de los metales depende de su especiación química que obedece, a su vez, a la composición mineralógica y elemental, y a las propiedades fisicoquímicas que esta confiere al sedimento.

En este sentido, merece una mención especial el tamaño de partícula, cuyas características determinan la implicación de los sedimentos en la movilización, deposición y dispersión de los metales en los sistemas acuáticos. Aunque no ha sido el objetivo de esta Tesis profundizar en los procesos hidrológicos que gobiernan la dinámica de los sedimentos, el estudio de la influencia de la estacionalidad de las condiciones hidrológicas del río sobre la migración de los metales asociados a las partículas en suspensión ha evidenciado un mayor riesgo ecológico y humano en la época de estiaje, debido fundamentalmente a la resuspensión de las partículas más finas del sedimento, cuya capacidad de acumulación de metales es superior a la de las partículas mayores.

Asimismo, se ha observado una ciclicidad en la función del sedimento, que pasa de ser fuente a sumidero de metales en condiciones de estiaje. Este conocimiento puede ser crucial para el desarrollo sostenible de un ecosistema urbano también en el marco del cambio climático, dado que los escenarios hidrológicos futuros predicen períodos de sequía más largos con un menor número de eventos de precipitación, aunque sí más torrenciales.

10.2. Recomendaciones

Tal y como evidencia esta Tesis Doctoral, el Sedimento puede aportar una valiosa información prospectiva sobre el estado y evolución de la calidad de las aguas en los ríos, lo que supone un gran avance en la gestión y evaluación del estado ecológico de una cuenca hidrográfica.

Si bien la Agencia Vasca del Agua–Ur Agentzia (URA), a quien incumbe la gestión de la parte de la demarcación hidrográfica del Cantábrico Oriental competencia de la Comunidad Autónoma del País Vasco, incluye la matriz sedimento en el análisis del cumplimiento de las NCA para sustancias prioritarias y otros contaminantes, sería beneficioso que extendiese su incorporación a toda la red de seguimiento del estado ecológico de los ríos. Huelga decir que esta y las sucesivas recomendaciones son extensibles a otros organismos y entidades tanto del ámbito estatal como del europeo e internacional.

Además, sería aconsejable determinar una periodicidad del muestreo de sedimentos que permita lograr un nivel aceptable de fiabilidad y precisión. A la vista de la ciclicidad del comportamiento del sedimento con la estacionalidad, se propone aumentar la frecuencia mínima actual (anual) establecida en el Real Decreto 817/2015 a, al menos, una frecuencia bianual. En este caso, el requisito fundamental para fijar las fechas en las que se efectúe el muestreo deberá ser la selección de dos épocas con condiciones hidrológicas del río marcadamente distintas para facilitar, con el tiempo, un análisis más fiable del estado ecológico y la sostenibilidad de una cuenca hidrográfica ante un posible cambio climático.

Obviamente, y en base a las conclusiones comentadas en este mismo capítulo, se recomienda la consideración, al menos, de los indicadores de calidad biológica (abundancia, biodiversidad y metabolismo de las comunidades microbianas), química (contenido total, especiación química y bioaccesibilidad de metales) y fisicoquímica (p.ej., tamaño de partícula, composición mineralógica y elemental, o contenido en materia orgánica y nutrientes) del sedimento evaluados en este trabajo. A este respecto, surgen dos nuevas recomendaciones:

1) Aunque este estudio se ha centrado especialmente en la comunidad bacteriana desnitrificante, resulta del todo necesario tener en cuenta la enorme biodiversidad y versatilidad metabólica de estas comunidades para el desarrollo de futuras investigaciones con un mayor grado de detalle. Ciertamente, existe una amplia variedad de procesos en cada ciclo biogeoquímico y conocer las distintas vías alternativas que configuran estos ciclos es de suma importancia. En el caso de esta Tesis Doctoral, y aunque no era uno de los objetivos, se han encontrado evidencias de la existencia de vías alternativas a la desnitrificación, como es el caso de la reducción desasimilatoria del nitrato a amonio (en inglés, dissimilatory nitrate reduction to ammonium (DNRA))

o la reducción del nitrato asociada al ciclo del azufre, procesos cuyo análisis detallado aumentaría el conocimiento que se tiene de la eliminación biológica de nitratos en los sedimentos de la cuenca del río Deba. Asimismo, sería recomendable que se tuvieran en cuenta otros ciclos biogeoquímicos, como el del carbono. La función de la materia carbonatada como fuente de electrones de las bacterias heterótrofas sugiere un acoplamiento de los ciclos del carbono y del nitrógeno, cuyo conocimiento, aún hoy reducido (Abril et al., 2019), es clave para un estudio exhaustivo de la comunidad bacteriana desnitrificante.

2) Si bien se ha puesto especial énfasis en los metales, es evidente que el control de la potencialidad tóxica de los sedimentos y, por tanto, determinación de la bioaccesibilidad de distintas especies acumuladas en los mismos, debe extenderse a todas las sustancias contaminantes cuyo seguimiento se establece de conformidad con las NCA recogidas en el Real Decreto 817/2015. Lógicamente, las normativas por las que se establecen estas NCA sufren continuas modificaciones como resultado de la incorporación de nuevas especies a la lista de sustancias contaminantes.

De manera específica se debe señalar, como recomendación, la consideración de los contaminantes emergentes, entre ellos, los fármacos. Aunque cuentan con un desarrollo de la investigación menor que otros contaminantes convencionales, su impacto sobre los ecosistemas y la salud pública se considera crónico debido a su bioacumulación en algunos niveles de la cadena trófica, su capacidad de alterar la actividad enzimática del microbiota o de propiciar la aparición de microorganismos resistentes, entre otras problemáticas (Jiménez Cartagena, 2011). Dada su persistencia en los sistemas acuáticos y su gran impacto sobre la salud ecosistémica y humana, estudios recientes han empleado la ocurrencia de fármacos en el agua y los sedimentos de río como indicador de la contaminación procedente de estaciones depuradoras de aguas residuales, entre otras fuentes (Bujagić et al., 2019; Čelic et al., 2019).

10.3. Referencias

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"In order to seek truth, it is necessary once in the course of our life to doubt, as far as possible, of all things"

René Descartes (1596-1650)

10. Conclusions and recommendations

It has been considered appropriate to individually include a detailed section with the specific conclusions regarding topics dealt with in each corresponding chapter of the present Doctoral Thesis. Therefore, only the main conclusions from this work are summarized here. In addition, various recommendations are included for its application and/or the development of future researches derived from this study.

10.1. Conclusions

In the field of the Integrated Water Resources Management (IWRM), the primary mission of managers is to achieve sustainable development of an urban ecosystem and meet together the environmental targets set out in the European Water Framework Directive (WFD, Directive 2000/60/EC). In this task, recognition of the susceptibility of an ecosystem is essential for effective decision-making. For this purpose, the present Doctoral Thesis helped us to stress the key role of sediments in the dynamics of pollutants within the aquatic environment and, thereby, in the evaluation of ecological status of a river basin, which was extremely valuable to assess the changes in the urban-industrial wastewater management undertaken during the last years in the Deba River catchment (Gipuzkoa, Basque Country).

The consideration of Sediment as an integrating element of the aquatic environment

(Water-Sediment-Biota) and its key role as source, sink and transport vector of pollutants were the cornerstone for the evaluation of the ecological status of the Deba River catchment. The ultimate goal was to suggest a complementary strategy to the monitoring programs established by the European and State legislation, mainly focused on the biological, chemical and physicochemical quality control of water.

In fact, an important conclusion of this research is the need to include Sediment in the monitoring programs for the evaluation of ecological status of a river basin. In this sense, the application of a multimetric index developed from chemical, physicochemical and biological quality indicators demonstrated that, at least, an annual sediment sampling provides a valuable information for prospective monitoring water quality, identifying those sites which could deserve special attention for planning future sustainability strategies.

Since the aim of this work was to evaluate the human impact on the ecological status of the Deba River catchment, the selection of the most appropriate sediment quality indicators became a critical step. Although the WFD (2000/60/EC) does not include the bacterial community as biological quality element for the classification of ecological status of a river, the study of its abundance, biodiversity (structural and functional) and enzymatic activity in sediments, specially focused on denitrifying bacteria, proved to be a suitable tool for predicting changes in river ecosystem functions and evaluating their vulnerability to treated and untreated wastewater discharges. It was observed that these discharges not only alter the spatial distribution and composition of bacterial communities involved in nitrogen and sulphur cycling —favoring possible greenhouse gas (N_2O) emissions as a result of an incomplete denitrification—, but also exceed their metabolic capacity to support the high nutrient loadings and to ensure river sustainability.

The implementation of corrective actions in the Deba River catchment, viz. the commissioning of four wastewater treatment plants (the last one in 2012) or the construction of a sewer from Ermua-Eibar to the Apraitz wastewater treatment plant in June 2014, promoted a considerable improvement of the chemical, physicochemical and biological status of various river sections. However, as the Basque Water Agency recently reported, some water bodies continue to be in serious breach of environmental objectives, given the bad ecological potential of the Ego stream, among others (URA, 2019). In accordance with

these observations, the results of this research (sampling campaigns from January 2015 to January 2016) demonstrated that effluents from the wastewater treatment plants and, mainly, untreated urban and industrial wastewater discharges increased organic matter, nutrient and metal content in water and sediments from the main channel and the tributaries of the Deba River catchment. In addition, the joint spatial evaluation of the chemical, physicochemical and biological quality of sediments (sampling campaign of October 2015) confirmed the current poor ecological status of some water bodies; specifically, the mid-low part of the main river and, primarily, the Ego stream.

Hence, another important conclusion of this research is the great potential of sediment bacterial community to assess the impact of different pollution sources on the ecological status of urban rivers due to their rapid response to any ecosystem perturbation. It, consequently, helps managers to immediately assess the suitability of the river basin management for planning future actions. Furthermore, the complexity and diversity of physical and biochemical processes in the Water-Sediment interface make extremely difficult to anticipate the physicochemical water quality related to organic matter and nutrients. However, the measurement of the enzymatic activity of sediment bacterial community is also a useful parameter to predict the evolution of these elements, since they constitute the source or product of their metabolism.

On the other hand, the determination of metal total content, chemical distribution and bioaccessibility as chemical indicators of sediments allowed us to identify the anthropogenic sources of metals. In addition, the ecological and human health risk assessment based on metal bioaccessibility was more accurate and reliable than the application of environmental quality standards (EQS), which mean their total concentration in sediments. In fact, this work demonstrated that the bioaccessibility and, therefore, the toxicity of metals depends on their chemical distribution which, in turn, is subjected to the mineralogical and elemental composition of sediments.

In this regard, the particle size distribution deserves special mention, since it determines the role of sediments in the mobilization, deposition and dispersion of metals within the aquatic environments. Although an accurate evaluation of the hydrological processes governing sediment dynamics was not a main objective of this work, studying

the influence of hydrological regime seasonality on the migration of metals associated to suspended particulate matter suggested that low flow season poses the highest ecological and human health risk. This might be primarily due to the resuspension of fine sediments with significantly higher metal content than bulk sediments.

Likewise, a cyclical behaviour of sediments was also observed since they act as sink for metals instead of metal source during dry periods. This knowledge could be crucial for sustainable development of an urban ecosystem also in the context of climate change, given that future hydrological scenarios establish longer dry periods between precipitation events.

10.2. Recommendations

As evidenced by the present Doctoral Thesis, Sediment provides a valuable information for prospective monitoring water quality in rivers, representing a great advance in the evaluation of ecological status and the management of a river basin.

The Basque Water Agency, responsible for the management of the river basins in the Eastern Cantabrian Hydrographic Demarcation within the competence of the Basque Country, includes Sediment in the analysis of compliance with environmental quality standards (EQS) for priority substances and other pollutants. However, it would be beneficial to consider the analysis of sediments in both chemical and biological quality monitoring programs for the evaluation of ecological status of rivers. Needless to say, this and the following recommendations are extensible to other State, European or international competent authorities.

In this sense, it would be also advisable to determine a suitable sediment sampling frequency to achieve an acceptable level of confidence and precision. In view of the cyclical behaviour of sediment as a function of seasonality, increasing the current minimum frequency (annual) established by the Royal Decree (RD 817/2015) to, at least, twice a year is proposed. In this case, the major requirement to set the times at which monitoring will be undertaken should be the selection of two periods with considerably different hydrological regime, to ensure a more reliable evaluation of the ecological status and sustainability of the river basin regarding climate change.

Obviously, based on the conclusions exposed above, the implementation of all biological (abundance, biodiversity and enzymatic activity of bacterial communities), chemical (metal total content, chemical distribution and bioaccessibility) and physicochemical (e.g., particle size distribution, mineralogical and elemental composition, or organic matter and nutrient content) sediment quality indicators evaluated in this work is suggested. In this regard, two new recommendations arise:

1) Although this study was especially focused on the denitrifying bacterial community, it is absolutely necessary to consider the enormous biodiversity and metabolic versatility of the entire microbial community for the future development of more accurate researches. Certainly, there is a wide variety of processes constituting each biogeochemical cycle and a deeper knowledge of alternative microbially mediated transformation of elements could be of crucial importance. In fact, the occurrence of other nitrate removal pathways like fermentative dissimilatory nitrate reduction to ammonium (DNRA) or sulfur-driven nitrate reduction seem to predominate over denitrification in some areas of the Deba River catchment. Likewise, consideration of other biogeochemical cycles, such as carbon cycling, is recommended for better comprehension of the denitrifying bacterial community. The role of organic matter as an electron donor for heterotrophic bacteria suggests a coupling of carbon and nitrogen cycling; however, its understanding is still quite limited (April et al., 2019).

2) In this work, special emphasis was placed on metals. However, the determination of the bioaccessibility in sediments and, therefore, the risk assessment of all pollutants subjected to the analysis of compliance with environmental quality standards (EQS) should be of concern. The number of pollutants listed in regulations are continuously increasing. Specifically, the consideration of emerging pollutants, such as pharmaceuticals, is recommended. Although little is known, their impact on ecosystem and human health is considered chronic due to their bioaccumulation in different trophic levels of the food chain, and their ability to alter the enzymatic activity of microbial community or to promote the emergence of resistant microorganisms, among others (Jiménez Cartagena, 2011). Given their persistence in aquatic environment and their great impact on ecosystem and human health, recent studies have used the occurrence

of pharmaceuticals in river water and sediments as chemical markers of wastewater contamination (Bujagić et al., 2019; Čelic et al., 2019).

10.3. References

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11

Appendix

Publications related to the present Doctoral Thesis

11.1 Articles

11.2 Contributions to congresses.

11. Appendix

11.1. Articles

Title:	Chemical and physiological metal bioaccessibility assessment in surface bottom sediments from the Deba River urban catchment: harmonization of PBET, TCLP, and BCR sequential extraction methods		
Authors:	Jessica Unda-Calvo, Miren Martínez-Santos, Estilita Ruiz-Romera		
Journal:	Ecotoxicology and Environmental Safety		
Year:	2017	**Volume:** 138	**Pages:** 260-270
Title:	Treated and untreated wastewater effluents alter river sediment bacterial communities involved in nitrogen and sulphur cycling		
Authors:	Miren Martínez-Santos, Anders Lanzén, Jessica Unda-Calvo, Iker Martín, Carlos Garbisu, Estilita Ruiz Romera		
Journal:	Science of the Total Environment		
Year:	2018	**Volume:** 633	**Pages:** 1051-1061
Title:	Links between data on chemical and biological quality parameters in wastewater-impacted river sediment and water samples		
Authors:	Miren Martínez-Santos, Anders Lanzén, Jessica Unda-Calvo, Iker Martín, Carlos Garbisu, Estilita Ruiz Romera		
Journal:	Science of the Total Environment		
Year:	2018	**Volume:** 19	**Pages:** 616-622
Title:	Implications of denitrification in the ecological status of an urban river using enzymatic activities in sediments as an indicator		
Authors:	Jessica Unda-Calvo, Miren Martínez-Santos, Estilita Ruiz-Romera, Juan Luis Lechuga-Crespo		
Journal:	Journal of Environmental Sciences		
Year:	2019	**Volume:** 75	**Pages:** 255-268
Title:	Evaluating the role of particle size on urban Environmental geochemistry of metals in Surface sediments		
Authors:	Jessica Unda-Calvo, Estilita Ruiz-Romera, Silvia Fdez-Ortiz de Vallejuelo, Miren Martínez-Santos, Ainara Gredilla		
Journal:	Science of the Total Environment		
Year:	2019	**Volume:** 646	**Pages:** 121-133

Title:	Multivariate statistical analyses for water and sediment quality index development: a study of susceptibility in an urban river
Authors:	Jessica Unda-Calvo, Estilita Ruiz-Romera, Miren Martínez-Santos, Maider Vidal, Iñaki Antigüedad
Journal:	Science of the Total Environment (In Press)
Year:	2019 **Volume:** **Pages:**

11.2. Contributions to congresses

Title:	Chemical and physiological metal bioaccessibility assessment in surface bottom sediments from the Deba River urban catchment: evaluation of urban and industrial inputs impact on sediments quality
Authors:	Jessica Unda-Calvo, Miren Martínez-Santos, Estilita Ruiz-Romera, Juan Luis Lechuga-Crespo
Congress:	The XV International Symposium on oceonagraphy of the Bay of Biscay (ISOBAY 15)
Presentation:	Poster **Date:** 22-24 June 2016 **Place:** Bilbao (Spain)

Title:	Spatial-temporal distribution of metal pollution in an urban-catchment case study: Deba River
Authors:	Juan Luis Lechuga-Crespo, Estilita Ruiz-Romera, Jon García-García, Miren Martínez-Santos, Jessica Unda-Calvo
Congress:	The XV International Symposium on oceonagraphy of the Bay of Biscay (ISOBAY 15)
Presentation:	Poster **Date:** 22-24 June 2016 **Place:** Bilbao (Spain)

Title:	Interrelation among metals and organic pollutants in a freshwater environment. Case study: The Deba river urban catchment (Gipuzkoa)
Authors:	Juan Luis Lechuga-Crespo, Estilita Ruiz-Romera, Jon García-García, Jessica Unda-Calvo
Congress:	The XV International Symposium on oceonagraphy of the Bay of Biscay (ISOBAY 15)
Presentation:	Poster **Date:** 22-24 June 2016 **Place:** Bilbao (Spain)

Title:	Effect of the implementation of a sewage treatment plant in the suspended sediments metal pollution during flood events in the Deba River urban catchment
Authors:	Jon García-García, Estilita Ruiz-Romera, Miren Martínez-Santos, Iñaki Antigüedad, Jessica Unda-Calvo
Congress:	The XV International Symposium on oceonagraphy of the Bay of Biscay (ISOBAY 15)
Presentation:	Poster **Date:** 22-24 June 2016 **Place:** Bilbao (Spain)

Title:	Metal Pollution Status of Suspended Particulate Matter and the Influence of Anthropogenic Activities of the Deba River Urban Catchment
Authors:	Jon García-García, Estilita Ruiz-Romera, Miren Martínez-Santos, Jessica Unda-Calvo
Congress:	18th International Conference on Heavy Metals in the Environment
Presentation:	Oral **Date:** 12-15 September 2016 **Place:** Ghent (Belgium)

Title:	Implications of denitrification in the ecological status of an urban river using enzymatic activities in sediments as an indicator
Authors:	Jessica Unda-Calvo, Miren Martínez-Santos, Estilita Ruiz-Romera, Juan Luis Lechuga-Crespo
Congress:	VII International Conference on Environmental Industrial and applied Microbiology (BioMicroWorld 2018 Conference)
Presentation:	Poster **Date:** 24-25 May 2018 **Place:** Torremolinos (Spain)

Title:	Do wastewater effluents alter river sediment bacterial communities?
Authors:	Miren Martínez-Santos, Anders Lanzé, Jessica Unda-Calvo, Iker Martín, Carlos Garbisu, Estilita Ruiz-Romera
Congress:	VII International Conference on Environmental Industrial and applied Microbiology (BioMicroWorld 2018 Conference)
Presentation:	Poster **Date:** 24-25 May 2018 **Place:** Torremolinos (Spain)

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Comencé en el mundo de la investigación científica en septiembre de 2013. Desde entonces hasta ahora, más de seis años de arduo pero gratificante trabajo —un Proyecto Fin de Carrera, un Proyecto Fin de Máster y una Tesis—, he conocido a multitud de personas que de una u otra manera han influido tanto en mi vida profesional como personal. A todas ellas gracias.

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Eskerrik asko denoi.

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La continua intensificación de la industria vasca ha permitido fortalecer el tejido empresarial en el que se sostiene una población en constante crecimiento. Sin embargo, este hecho ha generado múltiples presiones sobre el medio ambiente, llegando a comprometer el estado ecológico de los ríos, a lo largo de los cuales se asientan los mayores núcleos urbanos e industriales. En esta coyuntura se hace imprescindible la consecución de una armonización entre la protección del medio ambiente y un desarrollo regional sostenible. Con este propósito, se han acometido recientemente cambios en la gestión de las aguas residuales urbanas e industriales en la cuenca del río Deba (Gipuzkoa), una de las más contaminadas del País Vasco. A la degradación de la calidad de sus aguas, hay que añadir el impacto que dichos vertidos ocasionan en la calidad de los sedimentos, que actúan como fuente, sumidero y vector de transporte de contaminantes. Sin embargo, a pesar de que proporciona un registro histórico de la contaminación, la legislación europea, estatal y autonómica no otorga al Sedimento el mismo nivel de relevancia que al Agua en la evaluación del estado ecológico de las cuencas hidrográficas.

El objetivo principal de este trabajo fue remarcar el papel fundamental de los sedimentos en la dinámica de contaminantes en los ecosistemas acuáticos, y valorar el efecto de la gestión de vertidos de aguas residuales urbano-industriales sobre el estado ecológico de la cuenca del río Deba, empleando indicadores de calidad fisicoquímica, así como biológica.

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