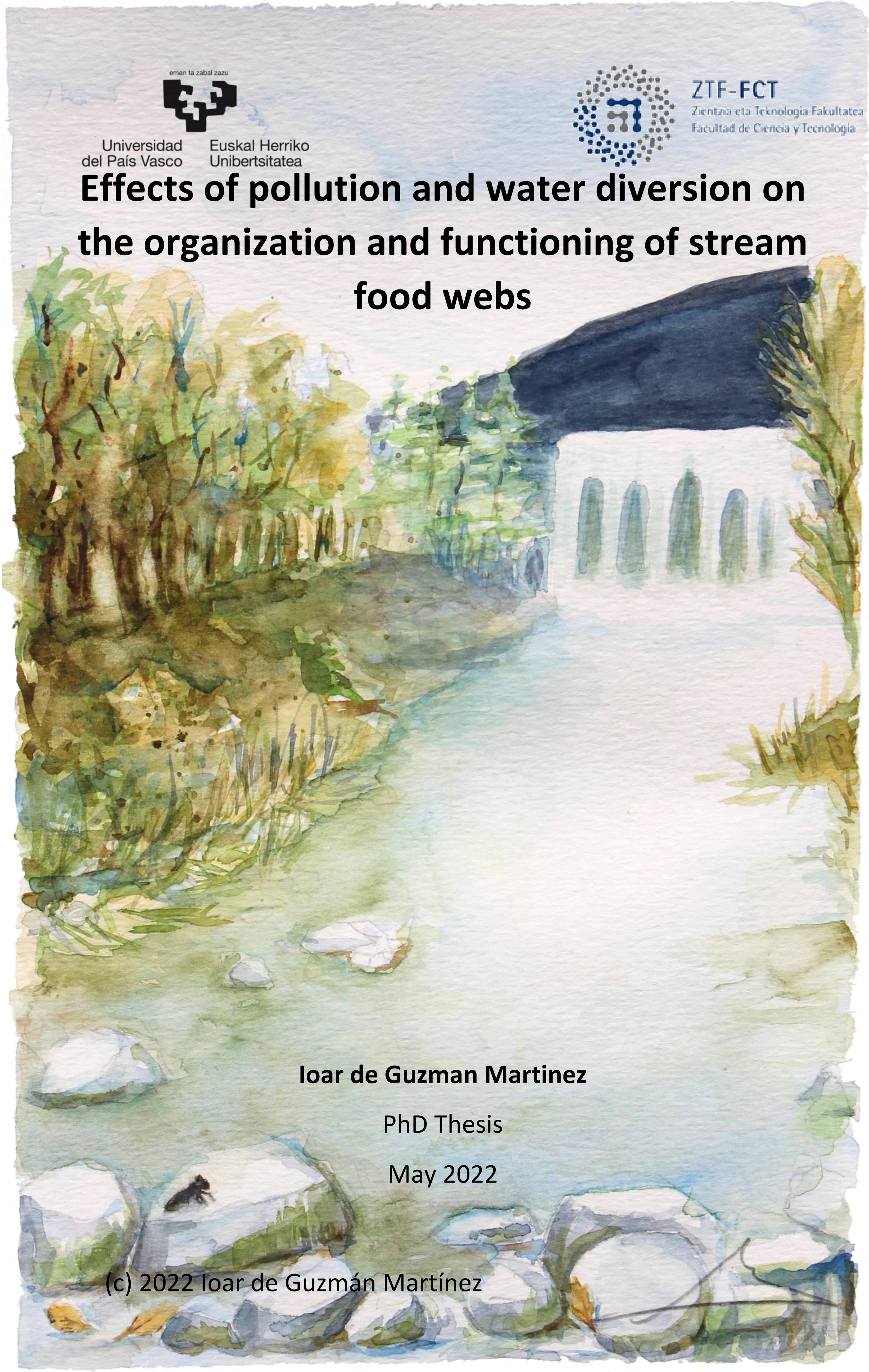

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Universidad del País Vasco
Euskal Herriko Unibertsitatea

ZTF-FCT
Zientzia eta Teknologia Fakultatea
Facultad de Ciencia y Tecnología

Effects of pollution and water diversion on the organization and functioning of stream food webs

Ioar de Guzman Martinez

PhD Thesis

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Supervised by Aitor Larrañaga and Jose M. Montoya

Cover image by Laura Barroso

"The history of life on earth has been a history of interaction between living things and their surroundings. To a large extent, the physical form and the habits of the earth's vegetation and its animal life have been molded by the environment. Considering the whole span of earthly time, the opposite effect, in which life actually modifies its surroundings, has been relatively slight. Only within the moment of time represented by the present century has one species—man—acquired significant power to alter the nature of his world."

Rachel Carson, Silent Spring

"*Biological diversity is the key to the maintenance of the world as we know it. Life in a local site struck down by a passing storm springs back quickly: opportunistic species rush in, to fill the spaces. They entrain the succession that circles back to something resembling the original state of the environment*"

Edward O. Wilson, The Diversity of Life

"*Be naive enough to start, stubborn enough to finish*"

Ross Edgley

Gurasoei, uneoro

eskaini didaten babesarengatik

Esker onak – Agradecimientos – Acknowledgements

Iritsi da tesia hasi nuenetik, duen garrantzia dela eta, idazteko zailena iruditzen zitzaidan atala idazteko unea. Hainbat izan zarete azken lau urteotako bidai honetan nire ondoan izan, eta helburu hau lortzen lagundu didazuenak. Ezagutzen nauzuenek badakizue zeinen zaila den niretzat sentimendu hauek idatziz adieraztea, baina zuei zor dizuet etapa honen amaierara iritsi izana. Lau urte luze izan dira, baina badakizuenez, inondik inora ez nuke egindako ibilbidea aldatuko. Une gogorrak egon badira ere, askoz ugariagoak izan dira momentu onak, eta zuei guztiei esker bidaia honetan asko ikasi eta disfrutatu dudala esan dezaket. Zuetako bakoitzari beraz, bihotzez, mila mila esker!

Lehenik eta behin Aitor, zuri eman nahi dizkizut eskerrak, niregan konfiantza eduki eta zurekin tesia burutzeko aukera emateagatik. Gradu amaierako lanetik tesiaren amaierara arte eskaini dizkidazun ordu guztiak eskertzeko idatzitako hitz guztiak nahikoak ez direla iruditzen zait, baina mila esker benetan erakutsi eta irakatsitako guztiarengatik. Zuzendari bikaina izateaz gain uneoro ondoan egon den bidelaguna ere izan zarelako, eskerrik asko! Muchísimas gracias también a tí Jose, por aceptar la propuesta de Aitor para dirigir esta tesis. Quiero agradecerte todo el tiempo dedicado y los consejos que me has dado. Sé que dirigir una tesis en la distancia no debe de ser sencillo, y aunque finalmente no he ido a Moulis más veces como habíamos hablado, te agradezco todas y cada una de las invitaciones además de la cálida acogida que me has dado en tu grupo. Gracias también por el sinfín de horas de reuniones que hemos tenido, de las cuales ni te imaginas lo mucho que he aprendido. Por eso, eskerrik asko biei bihotzez.

Eta nola ez, eskerrik asko zuei, laborategiko kideei, tesia burutzearen abentura zertan datzan ondo ulertzen duzuenei. Arturo, eskerrik asko taldean onartu eta proiektu interesgarri hauetan parte hartzeko aukera emateagatik, baita zure ezagutza gurekin elkarbanatu eta bidalitako lanak hain modu azkar eta sakonean begiratzeagatik ere. Vielen Dank auch dir, Dani, por transmitirnos la importancia de un trabajo meticuloso y bien ejecutado, por todos los consejos que me has dado y por la ayuda que me sigues proporcionando. Y ti Jose G. también muchas gracias, sobre todo por toda la ayuda procurada con los bichillos y la disposición de ayudar continuamente. Ahora a mí también me parece que los ceracleas y los ceratopogonidos son como los mapaches y las iguanas: parecidos solo si pones mucha voluntad. Asko izan zarete etapa honetan nirekin batera laborategi eta taldean ibili zaretenak, eta guztiengandik zerbait ikasi dudalako, eskerrik asko denei! Baina batez ere eskerrik asko zuei nexkak, zuen laguntasunik gabe bide hau zeharo desberdina izango zelako. Beteranen taldeari, Maite, Silvia, Olatz, laborategi eta landa lana burutzen irakasteagatik eta zuri batez ere Libe, nire erreferentea izateagatik, mila esker. Eskerrik asko zuei ere txikiñuelas, zuek gabe beteranen aurkako piragua lasterketan hain ondo pasatzerik edukiko ez nuelako, egunerokotasunean hainbeste barre egingo ez genuelako, porque sí, “Miss Dramones de la vida” gure montaña rusa etengabeak existituko ez liratekelako, eta gure etorkizuneko eta iraganeko “gu”-ak ezagutuko ez genituelako, gure “pomodoro time”-ak berdinak izango ez zirelako edo Lukenek gurekin hainbeste ordu emango ez zituelako; eta horrez gain, because we are doing a great job. Especialment gràcies a tu Vicki, per les nostres sortides multilingües a recollir fullaraca i els entrepans a migjan matí, per les classes de català i els mintzamenes d'euskera, per la infinitat d'hores de treball mà a mà i la nostra compenetració, per permetre'm donar-li caramels a Pau, per tot això i més, moltíssimes gràcies. Aipamen berezia ere egin behar diet Miren A eta Miren B-ri, dos Mirens y media-ren abenturak aspaldi hasi eta oraindik nire baitan daramatzadalako. A ti también Janire, la eficiencia personificada, gracias por ser el claro ejemplo de que podemos conseguir todo lo que nos propongamos. Buceta, Maria, Kelly, Lukas, Gorka, Ana, Josu, Leire,

Itsaso, Maialen, Laura, Jon, Beñat, Madalen, Nerea, Helena, Maialen, Marta eta laginketetan parte hartu duzuen biologo guztiei ere nire eskerrik beroenak. Estefy, Paula, vosotras también habéis sido parte de este trabajo, sobre todo gracias a ti Paula, por las múltiples horas de reuniones y trabajo conjunto durante la pandemia, por las infinitas horas puliendo los scripts mano a mano y, por ser tan buena compañera; ha sido un placer trabajar a tu lado. Miriam, Juan Pablo y Celi, pura energía, la calma y la dulzura en persona, a vosotros también gracias por los buenos momentos que hemos pasado. Kafe eta bazkalorduetako lagunak, zuek ere etapa honetan ezinbestekoak izan zarete, ia planak egitearen martxa berreskuratzen dugun.

I am also very grateful to you, Mario B., for giving me the opportunity of joining your group back in 2018 and helping me discovering the SIA world, and even after my stay in Magdeburg, for making the work together so easygoing. Ines, thank you as well for showing me what is “behind the scenes” of SIA and taking me in. Julia, Daniele and Nergui thank you for all the great moments during my stay Magdeburg. I’d also like to show my gratitude to Ulrich B. for accepting me at iDiv and giving me the chance of participating in all the group seminars and courses making the stay in Leipzig so interesting and profitable. Benoit G., thank you as well for all the time you spent with me, and for all the explanations and help you provided during these three months. Indeed, I am very grateful to the entire EcoNetLab for treating me as another member of the group, and for all the good moments we spent even in these weird days of the pandemics. Und auch dir, Vie, danke ich; du warst die beste Mitbewohnerin, den ich könnte in Leipzig haben. Thanks to you all! I learnt a lot during these stays and I spent such a good time.

Lau urte hauetan den-dena ere ez da lana izan. Asko izan zarete ondoan ibili zareten lagunak eta konturatu ez arren, nire ibilbidean zehar lagundu didazuenak. Bilboko erresistentziari eskerrak aisialdi momentutan tarteka tesiarekin ahazten laguntzeagatik zein nire ikerketaren gaia ulertzeko ahaleginak egiteagatik; tesiko ganba estresatuek ere zuen ardura eskertuko dutelakoan nago. Amaia, zuri ere eskerrik asko laginketetan parte hartu eta hainbat aurkezpenetako modelo izateagatik. Eta zuri ere Josh, Txinatik birusarekin batera etorri eta ordutik emandako momentu on guztiengatik mila esker; gogoratu orain Madrilera joan arren oraindik Auzotarron basoa betetzeke daukagula. Arratiatik mundurako nexkak, zuek gabe bide hau ere ez zen berdina izango. Dorle, eskerrik asko beti hor egoteagatik. Biologo onenei, karrera amaituta ere betiko laguntasuna erakutsi didazuenei. Batez ere zuri, Maialen, urrun ibili arren, beti ondoan zaitudala erakusten didazulako. Tesiaren ekuadorrean nengoela, 11 gaztek Ekuadorren bihotzera egin genuen txangoa bertako herritarrekin bizi eta euren errealitatea ezagutzeko. Egun horietan familiakoak bihurtu zineten guztiei ere zor dizuet honaino iritsi izana, bizitzan garrantzitsuak diren gauzetaz inoiz baino kontzienteago izan ginen bidaia hori ahaztezin bihurtu zenutelako. Meine Freunden von DF, ich danke euch auch für die “Dienstag auf Deutsch” Abends, por todas esas tardes que pasaron de ser para repasar las clases y si eso tomar algo a tardes para tomar algo, ver series y películas con doble subtítulo, cenar, video llamadas e incluso planear viajes y ya si eso para repasar alemán. Amaiur, energiaz eta zoriontasunez beteriko pertsona, zuri ere eskerrak eman behar dizkizut ondoan izan, zaletasunak elkarbanatu eta proiektuak aurrera eramateko indarra sustatzeagatik. Y a ti Adrian, mi compi de papeleos, gracias por ser últimamente mi “ángel de la guarda”, asegurarte no sólo de que me entere de convocatorias y de los plazos de subsanaciones sino también de todos los tramites de esta etapa final.

Gertukoak zaretenek ondo dakizue burua txoriz beterik edukitzen dudala maiz, eta hau lagun txorizale eta txoritologo guztiei zor diet. Txoriekiko zaletasuna, erreketan sartzen hasi nintzen momentu beretsutik datorkit, eta ordutik hainbat izan zarete bide honetan zehar lagundu nauzuenak. Bird Centerreko taldeari nire eskerrik beroenak zaletasun hau sustatu eta taldean beti besoak zabalik onartzeagatik. Eta ez bakarrik taldekoei, bertatik nire moduan pasa zareten guztiei ere, zuekin guztiekin, zuengandik eta bertako proiektuetan izugarri ikasi dudalako eta nire baitan daramatzadan hamaika momentu zoragarri eman dizkidazuelako. Hasiera hasieratik nire eraztunketa mentore izan zareten Edorta eta Luisi ere bihotzez eskerrik asko. Mila esker, Luis, zuri ere, Izaroko esperientzia zure ondotik bizitzen uzteagatik, eta bertan parte hartu duzuen Izarotar guztiei ere eskerrik asko azken lau udatan pasio hau elkarbanatu eta amets hau elkarrekin posible egin dugulako. Zoragarriak zarete! Rowan, I'm glad we shared such great moments inland and on our islands; thanks for being by my side all this time. Y a vosotros, los "babudos", en especial a ti Benito, por tenerme siempre tan presente. Y a Laura, amiga y artista, a quien le debo esta portada de tesis tan bonita. Eta nola ez, Leioaako kantera osoari, baina batez ere, Oier, Hector eta Iñigori, eskerrik asko estazioan eman ditugun ordu guztiengatik. Eta energiaz beterik zatozten baby ringers taldekoei ere, laguntasunaz gain, hainbat proiektu aurrera eramateko gogoak sustatzeagatik. Y gracias a Sergio y Bego, por darme la oportunidad de formarme con ellos y acogerme en el grupo siempre con los brazos abiertos. Gràcies també a tot l'equip de l'illa de l'Aire, especialment a en Santi i a en Raül per confiar en mi, acollir-me any rere any i donar-me l'oportunitat d'anar a aquest lloc meravellós (no només per ser la que pot entrar als nius de baldritjas a buscar els pollets), per ensenyar-me tant, i ser tan bons amb mi.

Etapa honetan zehar eduki nezakeen pixukiderik onena izan zarelako, Lore, zuri ere eskerrak eman behar dizkizut. Bi tesilari pandemian 24 orduz etxean giltzapeturik, zoramena ekar zezakeen, baina aldiz, inork baino hobeto bestearen egoera ezagututa, egun gogor hauei aurre egin genien aisialdi uneei frikismo puntu bat gehituta. Munitxen zabiltzala bisitaren bat egitera joan beharko naiz, faltan gehiegi bota aurretik! Igor, azken hileetan etxekoa ere bihurtu zarenari, izugarri eskertzen dizut eskainitako denbora eta tesiak dakartzan zailtasunak pazientziaz hartu izana. Hamaika izan dira, elkarrekin egon gintezen Bilbora egin dituzun ihesaldiak, eta hamaika ni animatu eta lasaitu beharreko uneak. Bihotzez eskertzen dizkizut ondoan emandako momentu guztiak, eginiko plan zoragarriak eta zure etengabeko poztasun eta umorea.

Familiari eta familiakoa zaren Gbenahitari, eta batez ere, Ama eta Aita, zuei. Gracias por estar siempre a mi lado, por confiar y creer en mí más de lo que yo lo hago. Gracias por guiarme y por apoyarme en todo momento, o por animarme cuando lo he necesitado. Porque cuando pintaban plantas íbamos a plantas, y ahora que pintan pájaros, vamos a pájaros. Gracias por estar siempre ahí, con vuestro apoyo siempre he conseguido llegar a la meta. Eskerrik asko bihotzez, maite zaituztet.

Lau urte hauetan ondoan izan zareten guztiei, mila esker.

I acknowledge the Basque Government for the financial support in terms of a predoctoral grant. This research was funded by the European Community´s 7th Framework Programme (GLOBAQUA; 603629-ENV-2013-6.2.1) and by the Spanish Department of Economy, Industry and Competitiveness through the project GL2016-77487-R (DIVERSION). I also acknowledge the financial support from the Basque Government (Consolidated Research Group IT951-16) and the Biscay Province Council (61/2015).

Content

Summary

The rising global human population and the intensification of economic activities have increased the demand of water, energy and other resources, forcing changes in the environment. Freshwaters, especially streams and rivers, are among the most threatened ecosystems on Earth due to the importance of water, a limited and fundamental resource. Pollution and water diversion are two of the most pervasive stressors affecting these ecosystems that often arise concurrently. Multiple stressors can interact and generate complex effects by amplifying or mitigating the individual effect of each stressor, and therefore, they have become of great concern for the alterations they can induce in ecosystem structure and functioning. In this line and considering the current biodiversity crisis that the global environmental change is driving, a call for food web analyses with a focus in interaction networks has been proposed for a better conservation of biodiversity and ecosystem processes due to the positive correlation between biodiversity and ecosystem functioning. This dissertation explored the effects of pollution water diversion on stream food webs, combining observational and manipulative field studies.

In the observational experiment (Chapters 1 and 2), we aimed at addressing the impacts of pollution and water diversion, on the structure and complexity of freshwater food webs. For this study, four rivers that differed in their ecological status and water quality were selected, which had a similar water diversion scheme. Using two different methodologies (i.e. stable isotope analyses and food web energy fluxes), food webs upstream and downstream the weirs were compared in a gradient of pollution, which ranged from low to moderate. Each stressor induced different changes at the base of the food web, with pollution increasing the availability of biofilm and water diversion reducing the stock of coarse detritus downstream from dams. In both chapters, the brown pathway showed a consistent negative response, decreasing with the reduction of detritus stock. However, contrary to what we expected, the relevance of the green food web did not increase along the gradient of pollution. Invertebrate community did not show large variations regarding density and diversity with increasing pollution, although communities became more homogeneous. Still, trophic diversity of the common taxa within rivers increased, indicating that they became more generalists. The observed alterations were larger in presence of both stressors, indicating that pollution exacerbated the effects of water diversion.

In the manipulative experiment, whole-ecosystem manipulation was performed (Chapters 3 and 4) to address the effects of point source pollution on food web properties. Following a BACI experimental design, which allows controlling spatial and temporal variability, alterations induced by a properly treated effluent were detected by means of the previously mentioned two methodologies. Although being highly diluted and showing low toxicity for microbial performance and detritivore growth, changes at the base of the food web were evident with the addition of the effluent and promoted the fluxes towards the green pathway. Despite the promotion of the green pathway, a reduction was observed in the brown pathway, paralleled to a decrease in the total energy fluxes and in trophic diversity. These responses were consistent to the observed decrease in invertebrate density and taxa richness, which also lead to an increased heterogeneity.

Overall, this dissertation showed that the studied stressors produced clear changes at the base of the food web, and that pollution enhanced the effects of water diversion along food webs. Additionally, it evidenced how the same stressor can produce contrasting patterns in the organization of food webs, which could be a consequence of different interaction strengths between the components of food webs.

Laburpena

Azken hamarkadetan jazotako munduko biztanleriaren emendioak eta jarduera ekonomikoen areagotzeak, uraren, energiaren eta beste baliabide batzuen eskaria handitu dute, ingurumenean aldaketak eraginez. Ur gezetako sistemak, errekak eta ibaiak batez ere, Lurreko ekosistema mehatxatuenetarikoak dira uraren garrantzia dela-eta. Izan ere, ura oinarrizkoa den baliabide mugatua da. Kutsadura eta ur ustiaketa dira ekosistema horietan ohikoak diren eta sarritan batera agertzen diren bi estres-faktore. Sistema bati eragiten dioten estresore anitzek efektu konplexuak sortu ditzakete, elkarren arteko eraginen ondorioz eragile bakoitzaren inpaktua emendatu zein arindu baitaitezke. Ekosistemen egituran eta funtzionamenduan eragin ditzaketen aldaketak direla-eta, egun kezka handia sortzen dute estresore anitzek. Honen ondorioz eta ingurumen-aldaketa globalak eragindako biodibertsitate-krisia kontuan hartuta, tesi honetan elkarrekintzatan oinarritutako sare trofikoak aztertzea planteatzen da. Lan honek, ur erauzketek eta kutsadurak ibaietako sare trofikoetan duten eragina aztertzen ditu, behaketa eta manipulazio bidezko landa esperimentuak konbinatuz.

Behaketa-esperimentuan (1. eta 2. kapituluak), kutsadurak eta ur erauzketak ibaietako sare trofikoen egituran eta konplexutasunean duten eragina ikertu zen. Horretarako, egoera ekologiko eta uraren kalitate desberdina duten lau ibai aukeratu ziren, zeintzuek antzeko ur desbideratze eskema duten. Bi metodologia desberdinen bidez (isotopo egonkorren azterketa eta sare trofikoetako energia-fluxuen estimazioa), presetatik gora eta behera dauden sare trofikoak konparatu ziren, ibaiek sorturiko kutsadura-gradientea kontuan hartuta. Estresore bakoitzak hainbat aldaketa eragin zituen sare trofikoaren oinarrian: kutsadurak biofilmaren eskuragarritasuna emendatu zuen, eta ur detrakzioak detritu lodiaren erreserba murriztu zuen presetatik behera. Bi kapituluetan, bide arreak erantzun negatibo sendoa erakutsi zuen, detritu-stockaren murrizketarekin bat zetorrena. Halere, espero ez bezala, kutsadura-gradientean zehar elikadura-sare berdearen garrantzia ez zen handitu. Ornogabeen komunitateak ez zuen aldaketa handirik jasan kutsaduraren emendioarekin dentsitateari eta dibertsitateari dagokienez, nahiz eta komunitatea homogeneoagoa bihurtu zen gradientean zehar. Dena den, ikerketa eremuan amankomunak ziren taxonen dibertsitate trofikoa handitu zen, dieta jeneralistago baterako trantsizioa adieraziz. Bi estresoreen elkarrekintzak aldaketak nabarmendu zituen, kutsadurak ur desbideratzearen eraginak areagotu zituela azpimarratuz.

Bigarren esperimentuan (3. eta 4. kapituluak), ekosistema osoa manipulatu zen kutsadura puntualak sare trofikoen ezaugarrietan dituen ondorioak aztertzeko. BACI (Before-After, Control-Impact) diseinu esperimentalak espazio eta denboran aldakortasuna kontrolatzea ahalbidetzen du. Diseinu esperimental honen bidez, tratamendu tertziarioa jasandako efluenteak sare trofikoan eragindako alterazioak antzeman ziren arestian aipatutako bi metodologiak erabiliz. Nahiz eta gehitutako efluentea oso diluituta egon, eta aurretik burututako ikerketetan mikroorganismoen jardueran eta detritiboroen hazkuntzan toxikotasun txikia erakutsi, sare trofikoaren oinarrian aldaketa nabarmenak eragin zituen. Biofilm kopurua handitzeak bide berdeko fluxuak sustatu zituen. Dena den, emendio hori gertatu arren, bide arrea murriztu eta, aldi berean, energia-fluxu totalak eta dibertsitate trofikoa gutxiagotu ziren. Erantzun horiek ornogabeen dentsitatean eta taxonen dibertsitatean hautemandako murrizketekin bat etorri ziren, aldi berean komunitatea heterogeneoagoa bihurtzea eragin zuena.

Tesiak, aztertutako estresoreek sare trofikoetan aldaketa nabarmenak eragin ditzaketela nabarmentzen du sare trofikoaren oinarrian gertatzen diren aldaketen ondorioz, eta kutsadurak ur ustiaketaren eraginak areagotu ditzakela erakusten du. Gainera, estresore berberak sare trofikoen antolamenduan kontrako eraginak sor ditzakela erakutsi da, sare trofikoen osagaien arteko interakzio-indar desberdinen ondorioa izan daitekeena.

Resumen

El aumento de la población mundial y la intensificación de las actividades económicas han incrementado la demanda de agua, de energía y de otros recursos, induciendo cambios en el medio ambiente. Los sistemas de aguas dulces, especialmente los arroyos y ríos, son unos de los ecosistemas más amenazados de la Tierra debido a la importancia del agua, un recurso esencial y limitado. La contaminación y la detracción de agua son dos de los factores de estrés más frecuentes que afectan a estos ecosistemas y que a menudo aparecen de forma simultánea. Los múltiples estresores que afectan a un sistema pueden interactuar y generar efectos complejos amplificando o mitigando el efecto individual de cada uno de ellos, por lo que se han convertido en motivo de gran preocupación dadas las alteraciones que pueden inducir en la estructura y el funcionamiento de los ecosistemas. Por este motivo y teniendo en cuenta la actual crisis de biodiversidad provocada por el cambio ambiental global, en esta tesis se plantea el estudio de las redes tróficas con enfoque en las redes de interacción debido a la correlación positiva entre la biodiversidad y el funcionamiento de los ecosistemas. En esta disertación se exploran los efectos de la detracción de agua y la contaminación en las redes tróficas fluviales mediante la combinación de estudios de campo observacionales y manipulativos.

En el experimento observacional (Capítulos 1 y 2), se analizaron los impactos de la contaminación y la detracción de agua, en la estructura y complejidad de las redes tróficas fluviales. Para este estudio, se seleccionaron cuatro ríos que difieren en su estado ecológico y en la calidad del agua, los cuales constan de un esquema de desvío de agua similar. Utilizando dos metodologías diferentes (i.e. análisis de isótopos estables y flujos de energía de las redes tróficas), se compararon las redes tróficas aguas arriba y aguas abajo de las presas teniendo en cuenta el gradiente de contaminación de los ríos. Cada estresor indujo diferentes cambios en la base de la red trófica: la contaminación aumentó la disponibilidad del biofilm y la detracción de agua redujo la cantidad de detritus grueso aguas abajo de las presas. En ambos capítulos, la vía marrón mostró una respuesta negativa consistente, disminuyendo con la reducción del stock de detritus. Sin embargo, al contrario de lo que esperábamos, la relevancia de la red trófica verde no aumentó a lo largo del gradiente de contaminación. La comunidad de invertebrados no mostró grandes variaciones en lo que a densidad y diversidad se refiere con el aumento de la contaminación, aunque las comunidades se volvieron más homogéneas a lo largo del gradiente. Sin embargo, la diversidad trófica de los taxones comunes en el área de estudio aumentó, indicando la transición a una dieta más generalista. Las alteraciones observadas aumentaron en presencia de ambos estresores, lo que indica que la contaminación exacerbó los efectos de la desviación de agua.

En el segundo experimento, se realizó una manipulación de todo el ecosistema (Capítulos 3 y 4) para abordar los efectos de la contaminación puntual en las propiedades de las redes tróficas. Mediante un diseño experimental BACI (Before-After, Control-Impact), el cual permite controlar la variabilidad espacial y temporal, se detectaron las alteraciones inducidas por un efluente debidamente tratado siguiendo las dos metodologías anteriormente mencionadas. A pesar de que el efluente estuviese muy diluido y en estudios anteriores mostrarse una baja toxicidad para la actividad microbiana y el crecimiento de detritívoros, los cambios en la base de la red trófica fueron evidentes tras la adición del efluente. El aumento de la cantidad de biofilm promovió los flujos hacia la vía verde. Sin embargo, a pesar de éste aumento, se observó una reducción de la vía marrón al mismo tiempo que una disminución de los flujos energéticos totales y de la diversidad trófica. Estas respuestas fueron coherentes con la disminución observada en la densidad de invertebrados y en la riqueza de taxones, lo que también condujo a un aumento de la heterogeneidad.

Esta tesis demuestra que los estresores estudiados producen claros cambios en la base de la red trófica, y que la contaminación puede potenciar los efectos de la detracción de agua en ellas. Además, cabe resaltar que un mismo estresor puede producir patrones opuestos en la organización de las redes tróficas, lo que podría deberse a las diferentes fuerzas de interacción entre los componentes de las mismas.

General Introduction

Freshwater ecosystems facing global environmental change

Global human population has alarmingly increased (Bystroff, 2021) since the industrial development of the 20th century, which originated an increase in life expectancy and well-being (Steffen *et al*., 2011). This rise accelerated the demand of water, energy and other resources (Steffen *et al*., 2007; Grimm *et al*., 2008; Crist *et al*., 2017), forcing large changes in the environment over the last two centuries (Ripple *et al*., 2017), such as alterations in land use cover, biogeochemical cycles, climate and air quality (Boggs, 2016). With these global changes, human activities are intensely shaping the environment and affecting every ecosystem of our planet (UN Environment, 2019), as even the lowest pressures of different components of global change (e.g., warming, habitat loss, pollution), can result in large alterations in ecosystems (Hillebrand *et al*., 2020).

The tragedy of commons

Freshwaters, especially streams and rivers, are among the most threatened ecosystems on Earth (Dudgeon, 2019). Water is a limited and fundamental resource for biodiversity, and its use (e.g. consumption, contamination) conditions its availability for other users (Dudgeon, 2013), which makes these water bodies particularly vulnerable to “the tragedy of commons” (Dudgeon, 2019). Running waters are dynamic and complex ecosystems constituted by wet channels, flood plains, and riparian and hyporheic zones which host a large biodiversity (Sabater *et al*., 2009). They occupy the lowest-lying areas of the landscape and are tightly linked and respond to the activities in to the surrounding land (Sabater *et al*., 2009). Historically, human settlements have been developed along rivers due to the high water availability (Grimm *et al*., 2008; Sabater and Elosegi, 2009), and consequently have widely altered these ecosystems to satisfy their demands for domestic, agricultural and industrial uses (Albert *et al*., 2021). Considering the wide range of human induced stressors for freshwater ecosystems, a classification of threats has been proposed (Fig. 1), describing six main categories: climate change, flow regulation, pollution, land-use change, invasive species and overexploitation (Dudgeon, 2019). These threats do not affect ecosystems independently, as interactions among them are frequent (Dudgeon, 2019; Reid *et al*., 2019), which has created the umbrella term of "multiple stressors" (Ormerod *et al*., 2010; Sabater *et al*., 2018).

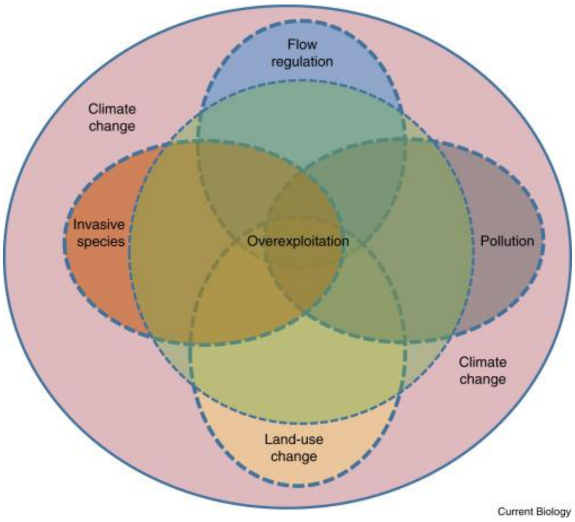

Fig. 1. Conceptual diagram of global threats to freshwater ecosystems. From Dudgeon *et al*., 2019.

Increasing pollution

Human population is not evenly distributed worldwide, as the 55% of the world's population is nowadays living in urban areas (United Nations, 2018). Changes in life habits lead by industrial and economic development are further increasing the amount of urban inhabitants (Steffen *et al.*, 2011) (Fig. 2), with predictions rising to 68% by 2050 due to migrations toward urban areas (United Nations, 2018). This increasing urbanization will keep incrementing the physical (e.g. hydrology or geomorphology) and chemical (i.e. pollution) alterations of river ecosystems promoting the "urban stream syndrome" (Walsh *et al*., 2005). The alterations in the environment during the last decades are a direct consequence of the fast growth of urban population. This growth makes pollution one of the main environmental concerns (Petrovic *et al*., 2013) due to the large amounts of biologically active compounds generated and released into freshwaters (Petrovic *et al*., 2013). Chemical pollution can consist of a wide range of contaminants (Schweitzer and Noblet, 2018), such as nutrients (Schweitzer and Noblet, 2018; Pereda *et al*., 2020), organic matter (Carey and Migliaccio, 2009), inorganic compounds (Petrovic *et al*., 2013) or organic micro pollutants (Rosi-Marshall *et al*., 2015; Solagaistua *et al*., 2018). The great compositional complexity turns the mixture into a multiple stressor on its own, as joint effects can be contrasting and convey surprising ecological consequences (Jackson *et al*., 2016). The variable composition and the concentration of the contaminants depend on different factors such as the origin of the compounds, which are determined by the dominant land-use (Posthuma *et al*., 2008), population density (Osorio *et al*., 2016) or the dilution in the receiving water body (Rice and Westerhoff, 2017).

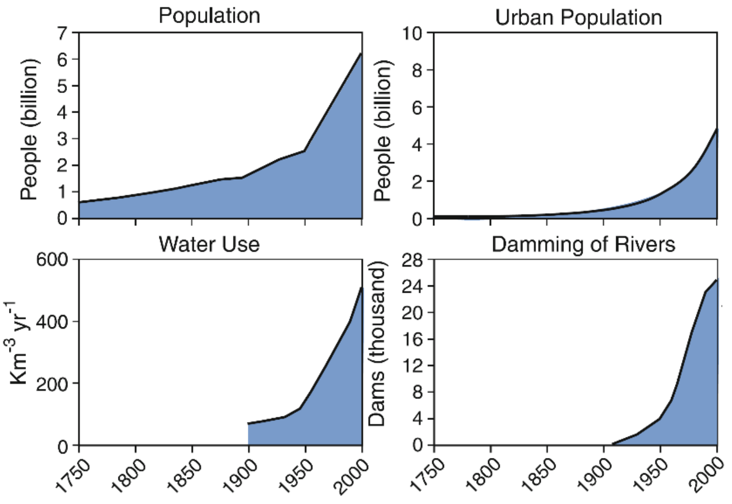

Fig. 2. The increasing rates of change in human activity since the beginning of the Industrial Revolution to 2000. Modified from Steffen *et al*., 2011.

Most of the rivers across Europe are affected by water pollution, although the main pollutant can change (Grizzetti *et al*., 2017) (Fig. 3). The increasing concern regarding the deterioration of these water bodies led to de development of new environmental laws during the last decades, such as the European Water Framework Directive (2000/60/EC) or their North American equivalents Clean Water Act and Canadian Environmental Protection Act. The proposed regulations aim at reducing anthropogenic pressures on these ecosystems and achieving a good ecological status of freshwaters, which is defined in terms of the quality of the biological community, the hydrological characteristics and the chemical characteristics. Additionally, further legislative frameworks, as the Urban Waste Water Treatment Directive (91/271/EEC), Nitrates Directive (91/676/EEC) or the Dangerous Substance Directive (67/548/EEC), have established emission limit values for specific contaminants to reduce the amount of pollutants entering these ecosystems after the collection and treatment of urban and industrial wastewaters. Consequently, in the last decades many wastewater treatment plants (WWTP) have been set into operation or upgraded (Serrano, 2007; Langergraber *et al*., 2018; Mas-Ponce *et al*., 2021) with the objective of complying with the current laws. These infrastructures are essential to improve sewage quality before releasing it into aquatic systems, as they are effective reducing effects associated to high pollution levels (Carey and Migliaccio, 2009). However, although WWTPs greatly reduce the amount of contaminants reaching aquatic ecosystems (Vaughan and Ormerod, 2012; Brion *et al*., 2015), treated sewage still consists of a complex mixture of pollutants, nutrients, and pathogens (Pascual-Benito *et al*., 2020). These compounds can produce contrasting responses on ecosystems, as some are simply toxic (Patel *et al*., 2020; Vasilachi *et al*., 2021), whereas others, such as nutrients, subsidize biological activity, although they can also become hazardous above a certain concentration (Carey and Migliaccio, 2009; Wang *et al*., 2019). Therefore, the effects of treated effluents on aquatic ecosystems strongly depend on effluent composition and on dilution rate, producing stronger alterations when they are poorly diluted in the receiving water mass (Carey and Migliaccio, 2009; Arenas-Sánchez *et al*., 2016).

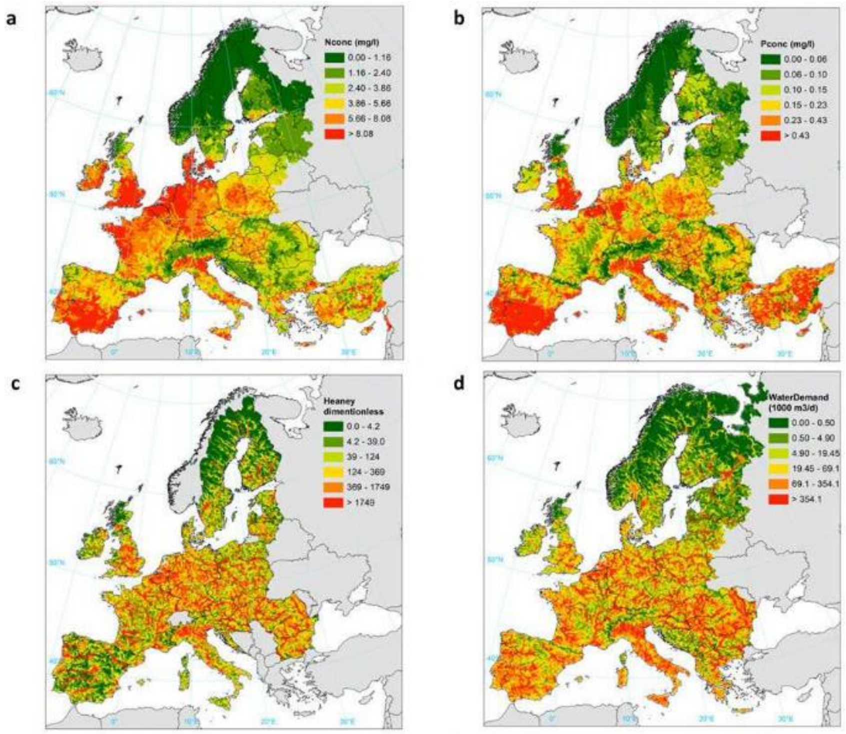

Fig. 3. Maps of pressures on European rivers. (a) Nitrogen concentration; (b) phosphorus concentration; (c) pollution from urban runoff and (d) water demand. From Grizzeti *et al*., 2017.

The rising water demand

Within the same global change scenario, the rising global human population and the intensification of economic activities have led to an increase in water demand (Crist *et al*., 2017; Ripple *et al*., 2017) (Fig. 2 and 3), which is projected to increase 50% more by 2050 (Leflaive, 2012). In order to satisfy this demand for domestic, agricultural, industrial and energetic purposes (Albert *et al*., 2021), rivers are increasingly being regulated (Belletti *et al*., 2020) (Fig. 2). Nowadays about half of all river reaches globally show truncated connectivity (Grill *et al*., 2019). Most of the barriers in European rivers (Fig. 4) are built to control and divert water flow, with weirs and dams as the most frequently built structures (30.5 % and 9.8 % respectively) (Belletti *et al*., 2020). Barriers are unevenly distributed, showing higher densities in densely populated areas and where an intense use of water is made (Belletti *et al*., 2020). Irrigation is among the most frequent water demanding activities, as over the 75% of the arable land is equipped for irrigation in semiarid regions (Siebert *et al*., 2013), and is predicted to increase under the current climate change scenario. The increasing energy demand is also a propellant of water diversion. To date, hydropower contributes 41.7% of the renewable electricity in the European Union and a 11.4 % of total electricity generation (Huđek *et al*., 2020). A revival in the construction of hydropower plants has been produced after the implementation of the European Renewable Energy Directive (2009/28/EC) (Wagner *et al*., 2019; Huđek *et al*., 2020). Its main goal is to reduce the emissions of greenhouse gases and therefore it establishes that renewable energies must constitute the 20 % of the gross energy consumption in the European Union. Consequently, the development of hydropower has been considered an alternative to mitigate, even reverse, climate change (Berga, 2016). Currently, there are over 21,300 hydropower plants in Europe, and additional 8,785 plants are planned or under construction (Schwarz, 2019). All these structures however, threaten freshwater biodiversity and the good ecological status pursued by the European Water Framework Directive, as hydro-morphological degradation is among the primary factors that prevent rivers from obtaining a good status. The impacts of large dams on freshwater ecosystems have been widely studied, and their effects are well described (e.g. Žganec, 2012; Aristi *et al*., 2014; Mor *et al*., 2018). However, the effects of the more frequent diversion or run-of-the-river schemes have received less attention as traditionally have been considered less threatening. These structures consist of a low weir or dam with little storage capacity and a water diversion canal that can divert some or most of the water from the by-passed reaches (Arroita *et al*., 2015).

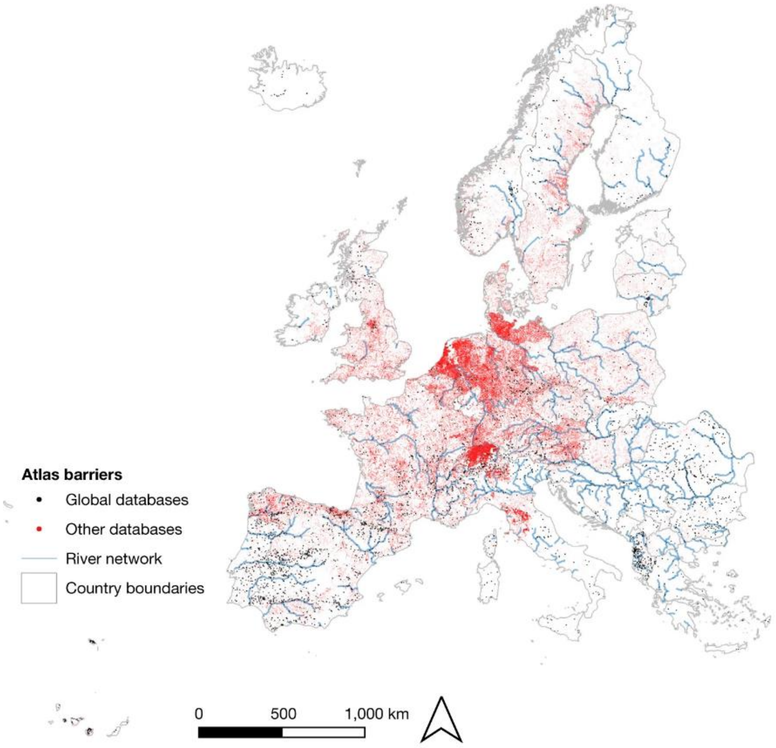

Fig. 4. Artificial instream barriers in Europe. From Belleti *et al*., 2020.

Alterations in ecosystem structure and functioning

At the time of assessing the effects of anthropogenic stressors on ecosystems, alterations on either structural or functional aspects can be studied (Sabater and Elosegi, 2013). Ecosystem structure includes both abiotic and biotic attributes that shape ecosystems, such as channel morphology, water physico-chemistry and the biological community (i.e. microbes, plants and animals) (Sabater and Elosegi, 2013; von Schiller *et al*., 2017). Ecosystem functioning refers to emergent ecosystem-level processes that regulate energy and matter fluxes in ecosystems due to the joint activity of organisms, including organic matter decomposition, nutrient cycling, biomass accrual, secondary production or ecosystem metabolism (von Schiller *et al*., 2017). Additionally, structure and functioning are related, as demonstrated by the relationship between diversity and ecosystem functioning (Reiss *et al*., 2009; Cardinale *et al*., 2012). Generally, biodiversity-ecosystem functioning (BEF) studies, which focus on detecting the causes by which alterations in biodiversity affect ecosystem processes, have shown consistent relationships between biodiversity and ecosystem functioning (Cardinale *et al*., 2011; O'Connor *et al*., 2017), with declines in biodiversity leading to losses in functioning (Loreau *et al*., 2001; Bestion *et al*., 2021). Moreover, these processes respond to specific environmental alterations (Young *et al*., 2008) and change at different spatial and temporal scales (Gessner and Chauvet, 2002), which has recently promoted the interest in assessing BEF across different spatial and temporal scales. These recent works show that, the importance of biodiversity for ecosystem functioning is larger as we consider longer temporal scales, larger spatial scales, and wider environmental fluctuations than the original, more simplistic experiments did (Isbell *et al*., 2018; Gonzalez *et al*., 2020; Bestion *et al*., 2021). As biodiversity and ecosystem functioning are linked to rates of processes that define ecosystem services necessary for humans (Balvanera *et al*., 2006; Tilman *et al*., 2014; Isbell *et al*., 2015), understanding the structure-functioning relationship becomes paramount.

The multiple effects of multiple stressors

Multiple stressors in freshwater ecosystems

Ecosystems usually are simultaneously influenced by multiple stressors (Ormerod *et al*., 2010; Sabater *et al*., 2018) and thus, the concern of unpredictable consequences on biological communities (Townsend *et al*., 2008; Sabater *et al*., 2016) and on ecosystem functioning (Piggott *et al*., 2015; Smeti *et al*., 2019) has drastically increased. This growing concern relies on the intricate way in which stressors can interact (Crain *et al*., 2008; Jackson *et al*., 2016; Orr *et al*., 2020) generating thus complex effects by amplifying or mitigating the individual effect of each stressor. Moreover, biological responses facing multiple stressors can vary (Berthelsen *et al*., 2018) and show contrasting patterns depending on target organisms (Matthaei *et al*., 2010; Elbrecht *et al*., 2016) and on the overall resistance of ecosystems (Jackson *et al*., 2016). Pollution (both point source and diffuse) and water diversion are two of the most prevalent stressors affecting freshwaters (Dudgeon, 2019; Reid *et al*., 2019). Some works have already identified pollution and hydrological alterations as a frequent paired-stressors in European rivers (Birk *et al*., 2018, 2020). However, as identifying and ranking key stressors supposes a challenge in multi-stressed systems (Dafforn *et al*., 2016), most of the studies usually address isolated impacts of individual stressors (O'Brien *et al*., 2019), which provides an overly simplified view of the pressures ecosystem are facing (Dafforn *et al*., 2016). Still, the increasing concern of the effects of multiple stressors have recently pushed the interest for this kind of studies.

Effects of pollution

Pollution (either point source or diffuse) is one of the most pervasive stressors in freshwater ecosystems (Malmqvist and Rundle, 2002; Reid *et al*., 2019) and appears frequently in conjunction with other stressors (Dolédec *et al*., 2021). Hydrological and water physico-chemical alterations are common impacts produced by point-source pollution in freshwater ecosystems. Effluent additions increase stream flows and water velocities, and can also alter stream channel width and depth, disturbing the natural flow regime of freshwater bodies (Merseburger *et al*., 2011; Plumlee *et al*., 2012). Effluents also alter water quality inducing shifts in the physicochemical characteristics (Hamdhani *et al*., 2020). For instance, decreases in water pH and dissolved oxygen levels are frequently reported in effluent-fed streams (Englert *et al*., 2013; Hamdhani *et al*., 2020). On the other hand, increases in temperature (Luthy *et al*., 2015), electrical conductivity and alkalinity (Hamdhani *et al*., 2020) are also commonly described, together with increases in pollutants (Solagaistua *et al*., 2018; Hamdhani *et al*., 2020) and nutrients (Carey and Migliaccio, 2009; Pereda *et al*., 2020).

All of these alterations can affect many aspects of biological communities such as the structure, complexity and diversity of food webs (García *et al*., 2017; Hamdhani *et al*., 2020) by inducing changes in features like growth, survival and reproduction of the different biotic groups (i.e. microbes, algae, invertebrates and vertebrates) (Northington and Hershey, 2006; Drury *et al*., 2013; Solagaistua *et al*., 2018). Nutrient pollution, which generally refers to the increased concentration of different forms of nitrogen and phosphorous (Schweitzer and Noblet, 2018), for example, reduces nutrient limitation of algal communities (Marcarelli *et al*., 2009) promoting primary production (Keck and Lepori, 2012). Additionally, nutrients can also increase the quality of organic matter (Cross *et al*., 2003, 2006), altering its processing (Halvorson *et al*., 2016, 2019). These changes at the base of the food web increase initial energy flow (Canning and Death, 2021) and can propagate towards higher trophic levels (Ardón *et al*., 2021) altering food web structure due to changes in taxonomic diversity and abundance, and increasing trophic diversity (García *et al*., 2017).

However, effects of pollution depend not only on the composition of the complex mixture but also on the characteristics of the receiving water body, where dilution capacity of freshwaters and the self-depuration capacity play an important role shaping the impacts produced on the structure and functioning of ecosystems (Carey and Migliaccio, 2009; Arenas-Sánchez *et al*., 2016; Rice and Westerhoff, 2017). In this regard, in the current global change scenario, in which the diversity of pollutants and the water scarcity are continuously increasing, the effects of pollution are exacerbating.

Effects of water diversion

Water diversion is another frequent stressor that menaces ecosystem integrity by affecting ecosystem structure and functioning (Arroita *et al*., 2015, 2017; González and Elosegi, 2021). Diverting part of stream flow through the canals reduces downstream discharge that results in a contraction of the wet channel (Arroita *et al*., 2017). Water physicochemical alterations have also been described downstream from low weirs such as increases in water temperature (Bae *et al*., 2016), water acidification (Rader and Belish, 1999; McIntosh *et al*., 2002) or alterations in oxygen saturation and conductivity (Rader and Belish, 1999). Moreover, the barriers disrupt longitudinal connectivity across the fluvial network limiting the dispersion of aquatic organisms (Brooks *et al*., 2018; Jones *et al*., 2020) and therefore, affect the structure of the communities (Carpenter-Bundhoo *et al*., 2020; Munasinghe *et al*., 2021). The above mentioned strong habitat contraction can also affect community composition (Stubbington *et al*., 2009), as ecosystem size is an important determinant of food web structure on freshwater ecosystems (McHugh *et al*., 2015; McIntosh *et al*., 2018). Additionally, water diversion schemes can modify the availability of basal food resources in rivers (Power *et al*., 2013). Coarse detritus for example, is reduced in the diverted sections compared to the upstream reaches due to its retention in the impoundments (Schmutz and Moog, 2018) and the transport through the diversion canals (Arroita *et al*., 2015). The altered availability of basal food resources can shape food webs through bottom-up mechanisms (Wallace *et al*., 1997; Biggs *et al*., 2000) and thus, modify energy and matter fluxes from detritus or primary producers, reconfiguring brown or green pathways of food webs. In the global change scenario, predictions ratify an increase of the detrimental effects of water diversion, as rainfall patterns change, mostly reduce, water availability in the different catchments (Reid *et al*., 2019).

The importance of food web studies

The global environmental change we are facing, has caused unprecedented extinctions of many taxonomic groups in the last decades, with these extinction rates becoming 100 to 1000 times higher than the background extinction rates (Pimm *et al*., 2014; Turvey and Crees, 2019). As species are pushed into extinction directly and indirectly, the fate of the remaining species is determined by the complex interactions between species (Montoya *et al*., 2006; Montoya, 2015). Both direct and indirect extinctions lead to simplified food webs that tend to be more inefficient in ecological process rates. In this line, as biodiversity has been positively linked to ecosystem functioning (Reiss *et al*., 2009; Loreau *et al*., 2021), the global biodiversity crisis concerns not only the loss of species but also the related consequences for ecosystem function (Thompson *et al*., 2012). Studies focusing on single trophic levels and their effect on ecosystem functioning are abundant in the literature (e.g. Woodward *et al*., 2012; Isbell *et al*., 2015), but multifunctional and multitrophic approaches have started to gain momentum (Barnes *et al*., 2014, 2018; Eisenhauer *et al*., 2019), which are highlighting hidden consequences of the loss of rare species (Loreau *et al*., 2001). Consequently, a call for food web analyses with a focus on species interaction networks has been proposed for a better understanding of biodiversity and ecosystem process dynamics (Harvey *et al*., 2017). In this regard, food web studies provide a quantitative framework that combine community ecology with ecosystem ecology (Thompson *et al*., 2012). The former focuses on patterns of species richness and community composition (species-centred approaches), whereas the later focuses on energy and nutrient fluxes through ecological systems (flux-centred approaches) (Thompson *et al*., 2012) (Fig. 5). Integrating these two approaches to expand BEF to food webs is challenging, but fundamental, which requires understanding species and populations characteristics, such as population-averaged body mass or abundance, of numerous species that conform complex natural communities (Brose and Hillebrand, 2016). At local scales, community biomass and species richness *per se* seem to be more important than the effects of species identity and community composition (Brose and Hillebrand, 2016), although keystone species that have a disproportionately large effects on some processes are also common (Woodward *et al*., 2008; Pérez *et al*., 2021). However, the current debate on the effects of global change on biodiversity loss (Norberg *et al*., 2022), highlights the importance of conducting BEF studies, and more generally, studies on the effects of global change components at larger spatial scales to discern whether species turnover maintains local and regional ecosystem functioning over time (Brose and Hillebrand, 2016).

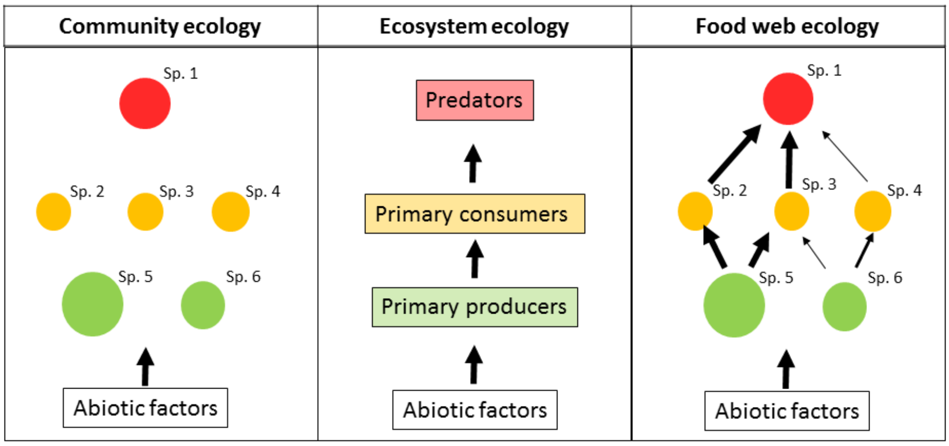

Fig. 5. Conceptual diagram of community, ecosystem and food web ecology. Modified from Thompson *et al*., 2012.

Traditionally food web reconstruction has been accomplished by observing evidences of interaction. Different approaches have been used to estimate this interaction, including non-destructive observations (Kytinou *et al*., 2020), gut content analyses (Pasquaud *et al*., 2007) or fatty acid analyses (Pasquaud *et al*., 2007; Ruess and Chamberlain, 2010) among others. However, fully characterizing food webs through these methods is a very laborious endeavor, since the identification of thousands of individuals might be needed to obtain a full set of feeding links. In the last decades, other methodologies have been developed for the investigation of food webs. DNA-based techniques for instance, have allowed the accomplishment of breakthrough dietary analyses in complex ecosystems (Roslin and Majaneva, 2016). The large availability of produced food web data has also allowed the expansion of food web studies based on stable isotope analyses, which require a basic previous knowledge of the system of interest (Phillips *et al*., 2014). Modeling has also become an important tool, which facilitates the reconstruction of food webs through the inclusion and combination of different drivers in sophisticated models (Kytinou *et al*., 2020).

Stable isotope analyses

Stable isotopes are the energetically stable atoms that do not decay due to the similar number of neutrons and protons (Fry, 2006). Lighter elements dominate biological compounds as consequence of the tiny mass differences that cause the isotopes to act differently in chemical reactions and physical processes (Hobson and Wassenaar, 2008). Hydrogen, carbon, nitrogen, oxygen, silicon and sulphur are the most common isotopes in ecological research (Fry, 2006). Stable isotope analyses of nitrogen and carbon provide a time-integrated assessment of trophic structure and interactions among species by estimating the relative trophic positions of consumers within a community and the contributions of distinct basal resources to their energetic needs (Peterson and Fry, 1987; Phillips, 2012). Due to fractionation differences (i.e. isotopic differences between the source and product compounds of a chemical transformation) among and within species (Vander Zanden and Rasmussen, 1999), stable isotopes have been used to quantify the trophic implications of a wide range of ecological processes (Fry, 2006; Layman *et al*., 2012). For instance, stable isotope analyses allow tracking different energy sources due to differences in carbon isotope fractionation among primary producers (Vander Zanden and Rasmussen, 1999), inferring the contribution of food sources to consumers' diet (Phillips, 2012). Relative position of consumers on a food web and food chain length (Post, 2002) can also be assessed, which gives information about the modifications that environmental drivers exert on trophic niches. In addition, carbon and nitrogen stable isotopes enable the characterization of trophic structure at the community-level, with metrics that use the position of each resource and consumer in the isotopic space and reflect the structure of the food web such as trophic diversity or redundancy (Layman *et al*., 2007; Jackson *et al*., 2011). Thus, stable isotope analyses have been successfully used for the assessment of anthropogenic pressures on food webs (Mancinelli and Vizzini, 2015).

Food web bioenergetics

One of the most common depiction of food webs is based on "Eltonian pyramids" constructed with abundances (Elton, 1927), which were later linked to transfer of energy from one trophic level to the next (Lindeman, 1942). These pyramids can easily display the overall efficiency of energy transfer on entire food webs or specific efficiencies on trophic levels. Size spectra, which is a translation of the Eltonian pyramid (Trebilco *et al*., 2013) is being used successfully in many fields of ecology, as it informs about how energy is shared and transported along the food web (White *et al*., 2007). Size spectra collates abundance and body size information of a food web, and the slope of the abundance-mass scaling relationship on a log scale informs on the trophic transfer efficiency in the community (Trebilco *et al*., 2013), being shallower when the energy is transferred more efficiently towards the higher trophic levels, and thus supporting higher abundance of large species (Woodward *et al*. 2005).

Other food web studies have adopted a more energetic perspective, characterized by the quantification of energy fluxes, which are a key aspect of ecosystem functioning, as a manner of describing ecological processes driven by trophic interactions among nodes in food webs (O'Neil, 1969). By means of these fluxes a quantification of functions such as herbivory and productivity can be carried out (Gauzens *et al*., 2019). Detailed estimation of these fluxes require a great effort and thus, mathematical proxies have been developed to estimate these fluxes. Additionally, there are allometric relationships between body size and individual growth, metabolic demands, individual longevity, population growth rate, and population density (Brown *et al*., 2004). Merging allometric theory to the calculation of energy fluxes, allows including wide ecological information such as biomass, metabolic demand, ecological efficiencies, or network topology to the calculation of energy fluxes in complex food webs (Gauzens *et al*., 2019). This approximation requires a large amount of previous knowledge on the system of interest to allow a realistic estimation of the energy transference. In freshwater ecosystems, a large number of food web studies have been carried out, providing detailed information regarding organisms' characteristics and interactions among them (Tachet *et al*., 2010; Gray *et al*., 2015). This amount of available information makes freshwater food webs particularly suitable to carry out calculations of energy fluxes.

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Objectives and thesis structure

The overarching objective of this dissertation is to assess the effects of pollution and water diversion, from the base of freshwater food webs to the entire organization (Table 1). To do so, we use an empirical approach that combines an observational and a manipulative field experiment. The **observational study** addresses the combined effects of pollution and diversion (Fig. 1), whereas the **manipulative study** focuses on the isolated effects of pollution (Fig. 2). In both empirical approaches, we use two different methodologies to investigate the changes on different components of food webs. By means of stable isotope analyses, we aim at addressing changes in **food web complexity**, with special focus on alterations on trophic pathways and on species' trophic niches. On the other hand, we use our own data to model food webs' bioenergetics and estimate **energy fluxes** as well as to assess changes in **community abundance and diversity**.

Specifically, this thesis tries to answer the following questions:

1. What are the interactive effects of pollution and water diversion on the complexity of food webs?
2. How does the combined effect of pollution and water diversion affect diversity at different spatial scales, community size-spectra and energy transfer in freshwater food webs?
3. How does pollution as a single stressor influence the complexity of stream food webs?
4. To which extent does pollution alter diversity, community size-spectra and the energy transfer on food webs?

To answer these questions, the dissertation is structured in four chapters (Table 1). The first two chapters focus on the observational study. In Chapter 1, stable isotope analyses are used to assess the impacts of pollution and water diversion on food web complexity, whereas the main axis of Chapter 2 involves estimations on energy fluxes. Chapters 3 and 4 are developed within the manipulative experiment in which we diverted part of a tertiary treated effluent into a small-unpolluted stream. Stable isotope analyses and estimations of energy fluxes are the main axes for Chapters 3 and 4 respectively.

Table 1. Main approaches addressed in this dissertation: effects of pollution, and pollution and water diversion on the response patterns of each variable.

		Observational experiment		Manipulative experiment	
	Stressor	Pollution & Diversion		Pollution	
	Objective	1	2	3	4
	Chapter	Chapter 1	Chapter 2	Chapter 3	Chapter 4
Availability of basal food resources	Biofilm biomass	✓		✓	
	Detritus stock	✓		✓	
Energy pathways	Green pathway	✓	✓	✓	✓
	Brown pathway	✓	✓	✓	✓
	Total energy fluxes		✓		✓
Abundance	Invertebrate density		✓		✓
	Size-spectra		✓		✓
Diversity	Taxa richness (α)		✓		✓
	Taxa richness (β)		✓		✓
	Shannon diversity (α)		✓		✓
	Shannon diversity (β)		✓		✓
Food web complexity	Maximum FCL	✓		✓	
	Trophic diversity	✓		✓	
	Trophic redundancy	✓		✓	

Fig. 1. Dams and studied reaches of the selected rivers ordered from lower to higher levels of pollution. Photographs provided by Vicki Perez.

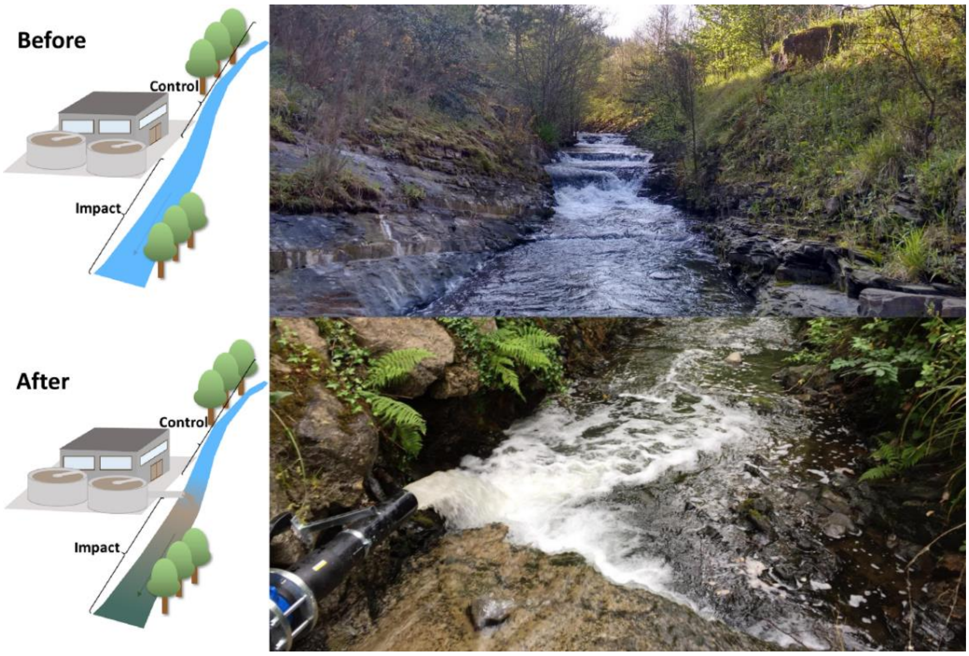

Fig. 2. Conceptual figure of the BACI (Before-After-Control-Impact) experimental design. The Control reach is upstream from the effluent pouring location. The Impact reach receives treated effluent during the After period. Photographs show the Control Reach and the effluent pouring pipe during a release ocassion. Photographs are provided by Miren Barrado and Arturo Elosegi.

Helburuak eta tesiaren egitura

Tesi honen helburu nagusia kutsadurak eta uraren erauzketak ibaietako sare trofikoetan eragiten dituzten inpaktuak ebaluatzea da, sare trofikoen oinarria eta antolamendu osoa kontutan hartuta (1. taula). Horretarako, hurbilketa enpirikoa gauzatu da, zeinetan behaketa eta manipulazio bidezko esperimentuak konbinatu diren. **Behaketa bidezko ikerketa**n kutsaduraren eta ur-ustiaketaren eragin bateratuak aztertzen dira; **esperimentu manipulatiboa**n ordea, kutsaduraren eragin isolatuaren ikerketa da ardatz nagusia. Esperimentu bakoitzean bi metodologia erabiltzen dira sare trofikoen osagai desberdinetan aldaketak aztertzeko. Isotopo egonkorren bitartez, **sare trofikoen konplexutasunean** gertatzen diren aldaketak aztertzen dira, bereziki bide trofiko eta taxonen nitxo trofikoei erreparatuta. Bestalde, **energia-fluxu**ak kalkulatzen dira ikerketan bertan lortutako organismoen gorputz-masen informazioa erabilita, ibaietako **komunitateen ugaritasunean eta aniztasunean** gertaturiko aldaketak ebaluatzearekin batera.

Tesi honek, zehazki, galdera hauei erantzun nahi die:

1. Zer eragin dauka kutsaduraren eta ur-desbideratzearen arteko elkarreraginak sare trofikoen konplexutasunean?
2. Nola eragiten dio kutsaduraren eta ur-desbideratzearen arteko elkarreraginak eskala desberdinetako dibertsitateari, komunitateen gorputz-tamainaren banaketari eta ibaietako sare trofikoetako energia-transferentziari?
3. Nola eragiten du kutsadurak, estres faktore isolatua izanik, ibaietako sare trofikoen konplexutasunean?
4. Kutsadurak zenbateraino aldatzen ditu dibertsitatea, komunitatearen gorputz-tamainaren banaketa eta sare trofikoetako energia-transferentzia?

Galdera hauei erantzuteko, tesia lau kapitulutan egituratu da (1. taula). Lehenengo bi kapituluak behaketa bidezko esperimentuari lotuta daude. 1. kapituluan, isotopo egonkorren analisiak burutu dira kutsadurak eta ur-desbideratzeak sare trofikoen konplexutasunean duten eragina ebaluatzeko. 2. kapituluan, berriz, energia-fluxuen azterketa burutzen da. 3. eta 4. kapituluak esperimentu manipulatiboaren baitan garatu dira, non tratamendu tertziarioa jasandako efluentea aldez aurretik kutsatu gabeko erreka batera isuri den. Isotopo egonkorren analisia eta energia-fluxuen estimazioa dira 3. eta 4. kapituluetako hurbilketa nagusiak, hurrenez hurren.

1 Taula. Tesi honetan jarraitutako hurbilketa nagusiak: kutsaduraren efektua, eta kutsadura eta ur-erauzketen arteko elkarreraginen inpaktuaren azterketa aldagai bakoitzaren erantzunean.

		Behaketa esperimentua		Esperimentu manipulatiboa	
	Estresorea	Kutsadura & Ur-erauzketa		Kutsadura	
	Helburua	1	2	3	4
	Kapitulua	1 Kapitulua	2 Kapitulua	3 Kapitulua	4 Kapitulua
Errekurtso basalen eskuragarritasuna	Biofilmaren biomasa	✓		✓	
	Detritu ugaritasuna	✓		✓	
Bide energetikoak	Bide berdea	✓	✓	✓	✓
	Bide arrea	✓	✓	✓	✓
	Energia fluxu totala		✓		✓
Ugaritasuna	Ornogabe dentsitatea		✓		✓
	Gorputz tamainen banaketa		✓		✓
Dibertsitatea	Taxon aberastasuna (α)		✓		✓
	Taxon aberastasuna (β)		✓		✓
	Shannon dibertsitatea (α)		✓		✓
	Shannon dibertsitatea (β)		✓		✓
Sare trofikoen konplexutasuna	Kate trofikoaren luzera maximoa	✓		✓	
	Dibertsitate trofikoa	✓		✓	
	Erredundantzia trofikoa	✓		✓	

Objetivos y estructura de la tesis

El objetivo general de esta tesis es evaluar los efectos que la contaminación y la detracción de agua inducen en las redes tróficas fluviales, desde la base hasta la organización completa (Tabla 1). Para ello, se utiliza una aproximación empírica combinando un experimento de campo observacional y otro manipulativo. El **estudio observacional** aborda los efectos combinados de la contaminación y la detracción de agua, mientras que el **estudio manipulativo** se centra en los efectos aislados de la contaminación. En ambos experimentos se utilizan dos metodologías diferentes para investigar los cambios en los distintos componentes de las redes alimentarias. Mediante el análisis de isótopos estables, se abordan los cambios en la **complejidad de las redes tróficas,** prestando especial atención a las alteraciones en las vías tróficas y nichos tróficos de los taxones presentes. Por otro lado, mediante los datos de masa corporal obtenidos en el propio estudio se estiman los **flujos de energía**, y se evaluan los cambios en **la abundancia y diversidad de las comunidades fluviales**.

En concreto, esta tesis trata de responder a las siguientes preguntas:

1. ¿Cuáles son los efectos interactivos de la contaminación y el desvío de aguas en la complejidad de las redes tróficas?
2. ¿Cómo afecta el efecto combinado de la contaminación y el desvío de aguas a la diversidad a diferentes escalas espaciales, a la distribución del tamaño corporal de las comunidades y a la transferencia de energía en las redes tróficas de agua dulce?
3. ¿Cómo influye único factor de estrés como la contaminación en la complejidad de las redes tróficas fluviales?
4. ¿La contaminación en qué medida altera la diversidad, la distribución del tamaño corporal de la comunidad y la transferencia de energía en las redes tróficas?

Para responder a estas preguntas, la tesis está estructurada en cuatro capítulos (Tabla 1). Los dos primeros capítulos se desarrollan en el estudio observacional. En el capítulo 1, se utilizan análisis de isótopos estables para evaluar los impactos de la contaminación y la desviación de agua en la complejidad de las redes tróficas, mientras que el capítulo 2 se centra en la estimación de los flujos de energía. Los capítulos 3 y 4 se desarrollan en el experimento manipulativo en el cual se desvió parte de un efluente terciariamente tratado a un pequeño arroyo previamente no contaminado. Los análisis de isótopos estables y las estimaciones de los flujos de energía son las aproximaciones principales de los capítulos 3 y 4 respectivamente.

Tabla 1. Principales enfoques abordados en esta tesis: efectos de la contaminación, y de la contaminación y detracción de agua simultáneamente en los patrones de respuesta de cada variable.

		Exp. observacional		Exp. manipulativo	
	Estresor	Contaminación & Detracción		Contaminación	
	Objetivo	1	2	3	4
	Capítulo	Capítulo 1	Capítulo 2	Capítulo 3	Capítulo 4
Disponibilidad de recursos basales	Biomasa de biofilm	✓		✓	
	Stock de detritus	✓		✓	
Vías energéticas	Vía verde	✓	✓	✓	✓
	Vía marrón	✓	✓	✓	✓
	Flujos totales de energía		✓		✓
Abundancia	Densidad de invertebrados		✓		✓
	Distribución de tamaño corporal		✓		✓
Diversidad	Riqueza taxonómica (α)		✓		✓
	Riqueza taxonómica (β)		✓		✓
	Diversidad Shannon (α)		✓		✓
	Diversidad Shannon (β)		✓		✓
Complejidad de redes tróficas	Longitud máxima de la cadena trófica	✓		✓	
	Diversidad trófica	✓		✓	
	Redundancia trófica	✓		✓	

Chapter 1

Water diversion and pollution interactively shape freshwater food webs through bottom-up mechanisms

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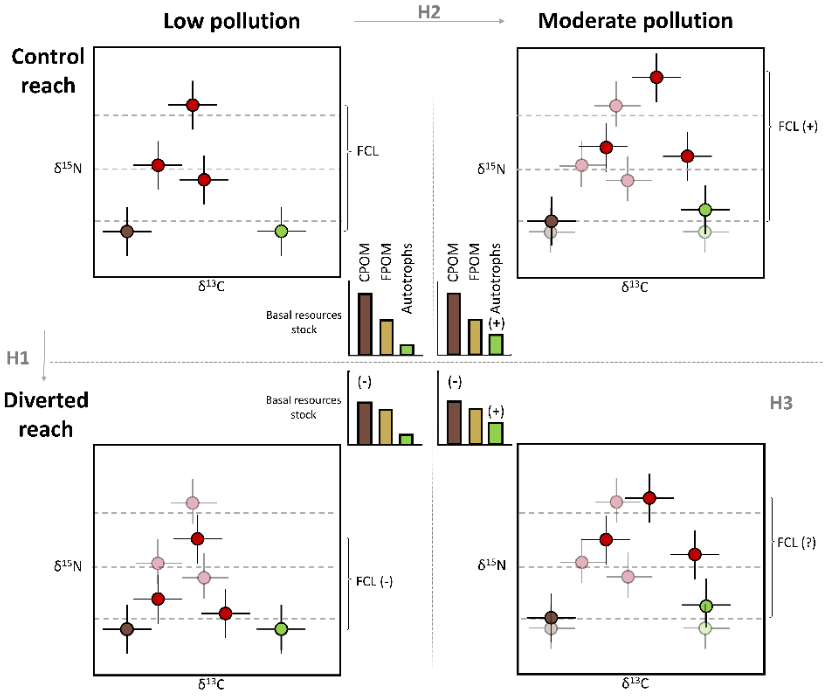
Global change biology (2021), 28(3), 859-876.

doi: 10.1111/gcb.16026

Abstract

Water diversion and pollution are two pervasive stressors in river ecosystems that often co-occur. Individual effects of both stressors on basal resources available to stream communities have been described, with diversion reducing detritus standing stocks and pollution increasing biomass of primary producers. However, interactive effects of both stressors on the structure and trophic basis of food webs remain unknown. We hypothesised that the interaction between both stressors increases the contribution of the green pathway in stream food webs. Given the key role of the high-quality, but less abundant, primary producers, we also hypothesised an increase of food web complexity with larger trophic diversity in presence of water diversion and pollution. To test these hypotheses, we selected four rivers in a range of pollution subject to similar water diversion schemes, and we compared food webs upstream and downstream of the diversion. We characterised food webs by means of stable isotope analysis. Both stressors directly changed the availability of basal resources, with water diversion affecting the brown food web by decreasing detritus stocks, and pollution enhancing the green food web by promoting biofilm production. The propagation of the effects at the base of the food web to higher trophic levels differed between stressors. Water diversion had little effect on the structure of food webs, but pollution increased food chain length and trophic diversity, and reduced trophic redundancy. The effects at higher trophic levels were exacerbated when combining both stressors, as the relative contribution of biofilm to the stock of basal resources increased even further. Overall, we conclude that moderate pollution increases food web complexity and that the interaction with water abstraction seems to amplify this effect. Our study shows the importance of assessing the interaction between stressors to create predictive tools for a proper management of ecosystems.

Key words: *Food web, water diversion, pollution, food web complexity, bottom-up mechanisms, stable isotopes*



Introduction

The rising global human population and the intensification of economic activities have increased the demand for water (Crist *et al*., 2017; Ripple *et al*., 2017), which is projected to increase over 50% by 2050 (Leflaive, 2012). In order to satisfy water demand for agriculture, industry and domestic use (Albert *et al*., 2021), rivers are increasingly being regulated by barriers, most of them built to control and divert water flow, with weirs (30.5%) and dams (9.8%) as the most frequently built structures (Belletti *et al*., 2020). These barriers disrupt connectivity across the fluvial network affecting dispersion of aquatic organisms (Brooks *et al*., 2018; Jones *et al*., 2020) and community structure (Carpenter-Bundhoo *et al*., 2020; Munasinghe *et al*., 2021). Water diversion driven by these infrastructures results in strong habitat contraction (Rolls *et al*., 2012), or even total loss of surface water flow (Steward *et al*., 2012; von Schiller *et al*., 2017). This also affects community composition (Stubbington *et al*., 2009), as ecosystem size is an important determinant of food web structure on freshwater ecosystems (McHugh *et al*., 2015; McIntosh *et al*., 2018). Moreover, water diversion can alter the availability of basal food resources in rivers (Power *et al*., 2013). For instance, coarse detritus is reduced in the diverted sections compared to the upstream reaches due to its retention in the impoundments (Schmutz and Moog, 2018) and the transport through the diversion canals (Arroita *et al*., 2015). Alterations in the availability of basal food resources modify river food webs through bottom-up mechanisms (Biggs *et al*., 2000; Wallace *et al*., 1997) and thus, modify energy and matter transfer from detritus or primary producers reshaping brown or green food webs, respectively.

Water diversion is usually accompanied by other stressors that simultaneously affect river ecosystems (Ormerod *et al*., 2010; Sabater *et al*., 2018). Multiple stressors often interact in an unpredictable way (Crain *et al*., 2008; Jackson *et al*., 2016; Orr *et al*., 2020) and generate complex effects by amplifying or lessening the single effect of each stressor, which depends not only on the interaction strength but also on the direction of the interaction (Piggott *et al*., 2015). Pollution is one of the most pervasive stressors in freshwater ecosystems (Malmqvist and Rundle, 2002; Reid *et al*., 2019) and appears frequently in conjunction with other stressors (Dolédec *et al*., 2021). Depending on their effects on biota, pollutants can be toxic (if they reduce biological activity at any concentration) or assimilable (if they subsidise biological activity at low concentrations but become toxic at high concentrations) (Odum *et al*., 1979). Nutrient pollution in aquatic systems, generally refers to the concentration of different forms of nitrogen (N) and phosphorous (P) (Schweitzer and Noblet, 2018), which frequently limit primary production (Elser *et al*., 2007; Tank and Dodds, 2003). Thus, enrichment of freshwaters with N and P can eliminate nutrient limitation of algal communities (Marcarelli *et al*., 2009) and increase biofilm biomass (Keck and Lepori, 2012). These changes in biofilm biomass increase initial energy flow (Canning and Death, 2021) and can be propagated towards higher trophic levels (Ardón *et al*., 2021) altering food web structure and increasing trophic diversity (García *et al*., 2017). In addition, as the energy loss within each trophic transfer limits species population size at high trophic levels (Hutchinson, 1959; Pimm, 1982), more productive ecosystems, which are not facing toxic consequences of eutrophication, should allow longer food chains. The main cause for this is that energy can more easily reach higher trophic levels (Pimm, 1982; Schoener, 1989) by narrowing the stoichiometric gap between consumers and their food resources (Mulder and Elser, 2009). Thus, longer food chains can be a product of a greater dietary generalism, which increases the trophic position of predators (O'Gorman *et al*., 2012).

There are a number of studies on the isolated effects of water diversion and water pollution on food web structure (e.g. Boddy *et al*., 2020; Walters and Post, 2008 and García *et al*., 2017;

Morrissey *et al.*, 2013; Price *et al.*, 2019). There are even a few studies that have addressed the joint effects of nutrient pollution and water diversion (e.g. Lange *et al.*, 2014) or flow reduction, a consequence of water diversion (e.g. Elbrecht *et al.*, 2016; Matthaei *et al.*, 2010) on river biota and functioning. Nevertheless, there is still a lack of studies assessing the effects of both stressors on the structure of food webs. Thus, the aim of this study is to assess the isolated and interactive effects of water diversion and water pollution on river food webs and trophic niche distribution. We hypothesise that (Fig. 1):

1. Water diversion will affect different dimensions of food web complexity in different ways. We expect that both the stock of coarse detritus and its contribution to the diet of primary consumers will decrease in diverted reaches, with a consequent increase in trophic diversity and a reduction of trophic redundancy by feeding more on the less abundant biofilm. However, reduced stock of basal resources will reduce food chain length (FCL).
2. Moderate pollution will increase food web complexity. We expect biofilm production and its contribution to the diet of primary consumers to increase with nutrients from moderately polluted waters, leading to an increase in FCL and trophic diversity.
3. The interaction between water diversion and pollution will increase even further food web complexity, as the reduction of coarse detritus stock will be accompanied by a larger biofilm availability. Consequently, the combination of water diversion and pollution will raise trophic diversity and reduce redundancy even further.

With this purpose, we analysed carbon and nitrogen stable isotopes, which provide a time-integrated measurement of trophic structure and interactions by elucidating the relative trophic positions of species in the food web (nitrogen isotope) and the relative contribution of different basal resources to the consumers (carbon isotope) (Peterson and Fry, 1987; Phillips, 2012).

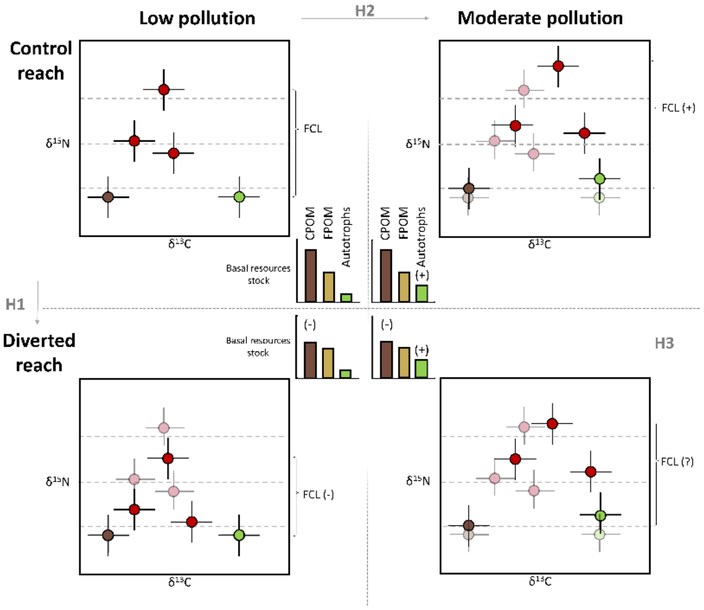


Fig. 1. Conceptual figure of the proposed hypotheses: H1 refers to the hypothesis regarding water diversion, H2 refers to the one related to the increase in pollution and H3 refers to the interaction between the two stressors. Control reach and diverted reach refer to the sampling sites above and below the weirs. Low pollution and moderate pollution is a simplification of the pollution gradient. CPOM and FPOM are coarse and fine detritus. FCL refers to food chain length. Basal food resources, coarse detritus, fine detritus and biofilm, are represented in dark brown, light brown and light green respectively in the figure, and consumers are represented in dark red. The food web arrangement of the C-N biplot from Control-Low pollution site is redrawn in ligther colours in the other biplots as reference. $\delta^{15}N$ indicates the

trophic position of each element of the biplot, whereas $\delta^{13}C$ informs about the relative proximity to the basal resources. (+) and (-) symbols indicate an increase or a decrease respectively.

Materials and methods

Sampling design and study sites

We selected four rivers within the temperate region of the northern Iberian Peninsula, which differed in their ecological status and water quality (Aguirre *et al*., 2017) (Table 1). None of the selected rivers showed a bad ecological status, which indicates that the pollution ranged from low to moderate. The cover and maturity of the riparian forests also differed between rivers (higher in Urumea and Leitzaran than in Kadagua and Deba), which was inversely related to the level of urbanisation (Table 1). The four rivers had a similar water diversion scheme, consisting of a low weir (3-6.5 m high) and a canal that can divert up to 90% of the river flow to hydropower. We defined two 100 m-long reaches in each river: a control reach upstream from the stagnant water retained by the weir and a diverted reach in the bypassed section immediately downstream from the weir.

Table 1. Main characteristics of the studied rivers. The total annual precipitation and mean annual air temperature are the average values for 2017 and 2018 (www.euskalmet.euskadi.eus). Ecological status and the Referenced Physicochemistry Index (RPI) for the period 2012-2016 are shown (Aguirre *et al*., 2017); the asterisk (*) indicates rivers with a heavily altered hydromorphology. Rivers are ordered following $\log_{10}$(TDN) values from left to right.

		Urumea	Leitzaran	Kadagua	Deba
Basin		Urumea	Oria	Kadagua	Deba
Coordinates of the dam	Latitude	43°12'53.5"N	43°07'57.6"N	43°13'37.9"N	43°09'37.6"N
	Longitude	1°54'16.7"W	1°56'13.4"W	3°00'58.8"W	2°24'08.6"W
Elevation (m asl)		69	354	37	122
Total annual precipitation (mm)		1838.6	2268.4	1288	1316.2
Mean annual air temp. (°C)		13.5	13.6	13.3	12.7
Upstream catchment area (km^2)		186.1	62.8	449	355.1
Land use (%) in upstream catchment area	Urban	0.1	1.1	2.5	4.6
	Agriculture	0.8	10.7	25.8	17.4
	Forestry	98.3	88.2	71.5	77.8
	Water	0.8	0.0	0.3	0.2
Ecological status		Good	Good	Good*	Moderate*
RPI		0.80 ± 0.01	0.79 ± 0.01	0.78 ± 0.01	0.68 ± 0.02
Maximum concession volume ($m^3 s^{-1}$)		5.8	3.0	4.0	5.0

Baseline data on water characteristics and basal resources

To set the baseline status of each river and include variability of water diversion and non-diversion periods, available information on water characteristics and basal food resources of three sampling campaigns (late spring of 2017, autumn of 2017 and late spring of 2018) were gathered. Water characteristics differed among rivers, but they were unaffected by water diversion (Table S1). According to the pollution gradient (which comprehended a wide range of pollutants apart from nutrients (see Aguirre *et al*. (2017)), but was represented in our study by the Total Dissolved Nitrogen (TDN) gradient; Table 2), Urumea was the least polluted river, followed by Leitzaran, Kadagua and Deba. This gradient correlated with the concentrations of most solutes, pH, conductivity, and temperature (Table S1, Fig. S1). Although we are aware that water nitrogen content was not the only driver shaping food webs and that the other physicochemical variables (e.g. temperature, oxygen or water flow) might also play a role, water TDN served as an effective surrogate when representing pollution in our systems. During these sampling campaigns, mean discharge and mean wet width showed a reduction (from 39.2% to 68.9% and from 4.6% to 25.9% respectively) downstream from the weirs (Table 2, Table S1). On each occasion, reach discharge (m3 s-1) was measured with an acoustic doppler velocity meter (ADV; Flow Tracker 2, SonTekHandheld-AD®, USA) through a cross-section. Wet channel mean width was obtained from equidistant transects every 10 m. Water was characterised by measuring temperature (°C), pH, electrical conductivity (µS cm-1) and dissolved oxygen saturation (%, DO) using hand-held probes (WTW Multi 350i and WTW 340i SET, WTW Wissenschaftlich, Weilheim, Germany; YSI ProODO handled; YSI Incorporated, Yellow Springs, OH, USA). Information on chemical composition of water samples were also gathered for each sampling occasion through analysis of filtered (0.7-µm pore size pre-combusted glass-fiber filters, Whatman GF/F, Whatman International Ldt., Kent, UK) and frozen (-20°C) water samples. The concentrations of nitrate (NO3-, mg L-1), sulfate (SO4 2-, mg L-1) and chloride (Cl-, mg L-1) in water samples were determined with capillary ion electrophoresis (Agilent G1600AX 3D, Agilent Technologies, Wilmington, DE, USA) (Environmental Protection Agency, 2007). Spectrophotometric (Shimadzu UV-1800 UVeVis, Shimadzu Corporation, Kyoto, Japan) methods were used to measure the concentration of soluble reactive phosphorus (SRP, µg P L-1) [molybdate method (Murphy and Riley, 1962)] and ammonium (NH4+, µg N L-1) [salicylate method (Reardon *et al*., 1966)]. Dissolved organic carbon (DOC, mg C L-1) and total dissolved nitrogen (TDN, mg N L-1) were measured by catalytic oxidation (Shimadzu TOC-L analyser coupled to a TNM-L unit).

We compiled the available information on the main basal food resources, to characterize the accessible food resources for the benthic community in each river and reach. Coarse detritus was collected by means of a Surber sampler (0.09 m2), and the organic matter retained on an 8-mm sieve was processed. Nine benthic samples were randomly collected in each reach on the aforementioned sampling campaigns. Information on fine detritus from two sampling campaigns (late spring and autumn 2017) was also gathered for each reach. Nine samples were randomly collected per reach in each sampling campaign using a sediment corer (surface 81.7 cm2). Both types of organic matter samples were oven-dried (70°C, 72h) and combusted (500°C, 4h) to determine their ash free dry mass (AFDM, g m-2). Information on biofilm biomass was gathered from a single sampling campaign in late spring of 2018, which was measured by means of a BenthoTorch fluorometer (Bentho Torch, bbe-Moldaenke, Germany) on 18 cobbles per reach. For details regarding the collected data, see de Guzman *et al*. (2021).

Table 2. Compiled mean and standard error of physicochemical descriptors in each reach of the studied rivers. TDN gradient score for each river is also indicated in the table. Rivers are ordered following $\log_{10}$(TDN) values from left to right.

	Urumea		Leitzaran		Kadagua		Deba	
	Control	Diverted	Control	Diverted	Control	Diverted	Control	Diverted
pH	7.65±0.09	7.54±0.08	8.13±0.05	8.14±0.02	8,16±0.13	8.27±0.10	8.14±0.19	8.15±0.04
Conductivity (µS cm^{-1})	75.13±5.89	74.97±6.32	243.00± 19.14	242.33± 19.64	586.00± 38.32	590.00± 45.51	505.00± 78.23	508.67± 61.48
Temperature (°C)	14.80±1.21	15.00±1.10	15.13±2.14	15.73±2.21	16.70±2.39	17.00±2.30	18.30±0.38	17.90±0.38
Dissolved oxygen (%)	108.90±5.77	108.60±6.82	103.30±6.58	102.3±0.88	97.00±0.05	103.9±1.93	115.40±0.91	105.80±4.68
Cl^- (mg L^{-1})	4.16±1.50	3.00±0.38	6.06±0.28	6.98±2.73	14.28±5.37	8.03±1.06	12.22±1.97	12.37±1.70
SO_4^{-2} (mg L^{-1})	2.55 ±0.91	2.1 ±0.46	7.07 ±0.95	7.18 ±3.17	35.83±10.93	20.32±1.06	19.85 ±5.99	20.02 ±5.70
NO_3^- (mg L^{-1})	1.58±0.67	1.04±0.22	2.53±0.11	2.81±0.94	3.56±2.70	0.98±0.17	2.13±0.38	2.22±0.17
NH_4^+ (µg N L^{-1})	15.65±2.69	16.68±5.61	14.32±4.70	13.90±5.38	56.72±8.61	51.53±9.82	27.02±7.07	24.50±6.75
DOC (mg C L^{-1})	1.91±0.14	1.91±0.48	3.39±0.15	3.97±0.39	6.40±0.52	7.89±0.83	5.67±0.96	6.14±0.26
TDN (mg N L^{-1})	0.85±0.06	0.85±0.04	1.11±0.10	1.26±0.17	1.54±0.10	1.47±0.11	1.94±0.28	1.94±0.35
SRP (µg P L^{-1})	6.78±1.12	9.92±1.73	32.18±11.42	29.04±9.58	31.59±11.10	28.10±9.90	54.11±9.90	49.25±16.45
Wet width (m)	21.57±0.57	15.98±0.75	10.12±0.42	9.66±0.40	20.88±0.79	16.89±0.74	20.61±0.79	16.58±0.44
Discharge (m^3 s^{-1})	4.60±1.43	1.71±0.23	1.67±0.62	0.9±0.68	2.57±1.22	0.80±0.10	1.55±0.63	0.94±0.13
TDN gradient (Log_{10}TDN)	-0.064±0.017		0.073±0.035		0.177±0.021		0.288±0.043	

Stable isotope analysis

A single food-web sampling campaign for stable isotope analysis (SIA) was carried out during late spring of 2018, a period when the flow differences between upstream and downstream reaches from dams are greatest because of low precipitations but still active diversion canals. All the available basal food resources were collected from each reach: biofilm, fine detritus and leaves of alder (Alnus glutinosa (L.) Gaertn) were present in every river and reach, while the gathering of filamentous green algae, bryophytes and macrophytes varied from reach to reach. Other basal resources, such as phytoplankton, were not collected due to their extremely low abundance in these river sections. Six composite samples of biofilm were collected in each reach by scrapping the whole surface of nine cobbles and collecting the slurry in filtered river water (0.7 µm pore size, Whatman GF/F). The remaining resources were individually gathered from the riverbed. Alder leaves found on the riverbed were collected as a representative of coarse detritus since they were the dominant leaf type found in these reaches. Alder leaves are a nutrient poor basal food resource compared to autochthonous resources (Cross *et al*., 2005); although, they show a relatively higher content of nutrients compared to leaf-litter of other woody plants (Kang *et al*., 2010). Macroinvertebrates were collected with a kick sampler (0.5 mm mesh aperture) in six transects along each reach. The six most common, but trophically diverse genera were collected after sorting and identifying them in the field: Baetis, Ecdyonurus, Echinogammarus, Ephemerella, Hydropsyche and Rhyacophila. Up to nine invertebrate samples per taxon were collected in each reach, each sample containing from one to 55 individuals depending on their body mass. When possible, the digestive tracts of the predators were removed, since gut contents can affect the isotopic signature of the sample (Mateo *et al*., 2008).

Fish sampling was conducted along the 100-m long reaches by depletion electrofishing with a backpack-electrofishing unit (Hans Grassl model IG2002/D30). Stop-nets were set upstream and downstream of the reaches. All cached fish were anaesthetised with MS-222, identified and up to five individuals per species in each reach were euthanized (reference number of the ethics commission: M20/2016/135). Samples of dorsal muscle were extracted in the field. All the samples were immediately frozen (-20°C) for processing and SIA.

Frozen samples were freeze-dried (VirTis Benchtop 2K) (from 12 to 72h depending on their water content), grounded (Resources in a ball-mill (Vibration mill MM301, Fisher Bioblock Scientific); Animal samples in a homogeniser (Precellys® 24, Bertin instruments)) and weighed (approximately 1 mg for invertebrates and fish, 10 mg for fine detritus and 2 mg for other basal resources) into tin capsules (Lüdiwiss Sn 98, 5 x 8 mm) for SIA. The Stable Isotope Facility of the University of California – Davis performed Carbon (C) and Nitrogen (N) stable isotope analyses on a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Results are expressed as the relative difference between ratios of samples and international standards (Pee-Dee Belemnite limestone formation for $\delta^{13}C$, atmospheric N for $\delta^{15}N$) and expressed in per mil delta notation [e.g. $\delta^{13}C = (Rsample/Rstandard-1) \times 1000$] (Fry, 2006) (Table S2). Analytical error (mean SD from in-house standards) associated with our sample runs was estimated at 0.2 ‰ for δ 13C and 0.3 ‰ for δ 15N. For details regarding the collected data, see (de Guzman *et al*., 2021).

Data treatment

TDN as a proxy of water pollution

In order to understand the correlations among the different physicochemical properties of the water and to define a gradient of pollution in the studied rivers, we carried out a principal component analysis (PCA) with the data of dissolved oxygen saturation, pH, temperature and solute concentrations (NO3-, SO4 2-, Cl-, SRP, NH4+, TDN and DOC) for each sampling campaign and reach. Due to the distribution of the components in the PC1 axis (Fig. S1, Table S1: correlation with PC1), the correlation of the variables with the TDN (Table S1: correlation with TDN), and the importance of nitrogen in determining the functioning of freshwater ecosystems (Dodds *et al.*, 1998), we used TDN as the covariate representing the pollution gradient in posterior statistical analyses ($\log_{10}$-transformed and centred into 0 using the average value of the four rivers) (Table 2).

Maximum FCL

Maximum FCL (the linear trophic distance between basal resources and top predators) in each site was estimated following the maximum trophic position convention, assessing top predators' trophic positions (TP) and comparing their $\delta^{15}N$ values to the mean $\delta^{15}N$ value of the basal resources at each sampling site (Cabana and Rasmussen, 1996): $TP_{top\ predator} = \frac{(\delta^{15}N_{top\ predator} - \delta^{15}N_{baseline})}{3.4} + \Lambda$. Where 3.4 is the trophic discrimination factor (TDF) of $\delta^{15}N$ (Post, 2002a; Vander Zanden and Rasmussen, 2001) and ʌ the trophic level of the baseline indicator, set as 1 because primary producers were used as the baseline. We obtained the maximum FCL from the mean TP values of the individuals with the highest TP in each reach (Table S3).

Contribution of resources to the diet of primary consumers

We used Bayesian Mixing Models to estimate the contribution of the various basal resources to the diets of the primary consumer invertebrates at each river and reach using the MixSIAR package (Bayesian Mixing Models in R; (Stock and Semmens, 2013). Autochthonous resources, fine detritus and alder leaves were treated as separate resources. Due to the very low and sparse distribution among sampling reaches biofilm, filamentous green algae, bryophytes and macrophytes were merged together into the autochthonous resources category. The models consider uncertainty and variation in consumers and TDF to generate a distribution of possible mixing solutions based on the available resources. MixSIAR also provides error terms that contemplate variation due to sampling processing and due to consumers' variability itself (i.e. individual differences in digestibility, assimilation efficiency and metabolic rates) (Stock and Semmens, 2016). We used TDF and uncertainties specific for aquatic invertebrates (0.1 ± 2.2‰ for $\delta^{13}C$ and 2.6 ± 2.0‰ for $\delta^{15}N$ (Brauns *et al.*, 2018)). Concentration dependence (Phillips and Koch, 2002) and a multiplicative error structure (Stock and Semmens, 2016) were also considered in the models. Posterior estimates of the proportional contribution of each resource to each consumer's diet were obtained for each reach. Consumer stable isotope data was previously checked for outliers through simulated mixing polygons (Smith *et al.*, 2013) with the packages sp (Pebesma *et al.*, 2012) and splancs (Bivand *et al.*, 2017). The method uses a Monte Carlo simulation to iterate Convex hulls ('mixing polygons') based on means and SD of source data and TDF. It applies the point-in-polygon assumption to test if source contributions can explain consumer's isotopic signature in the proposed mixing model. Following the recommendations by Smith *et al.* (2013), no data had to be excluded.

Community iso-space metrics

The trophic structure of the consumer community was estimated for each river and reach using the community-wide metrics described in Layman *et al*. (2007a) and Jackson *et al*. (2011). We considered three functional groups: primary consumers (Baetis, Ecdyonurus, Echinogammarus, Ephemerella and Hydropsyche), predatory invertebrates (Rhyacophila), and fish. Some metrics consider the distribution of the components of each community in the δ 13C - δ 15N space to inform about the trophic diversity within each food web. Mean distance to centroid (CD) is one of these metrics, which provides information on the trophic niche through the species distribution in the iso-space. Trophic redundancy was estimated using mean nearest neighbour distance (MNND) and standard deviation of the nearest neighbour distance (SDNND). MNND is the main metric representing trophic redundancy, which provides a measure of density and grouping of the community members. SDNND gives a measure of evenness of spatial density and packing. Smaller MNND represents food webs with taxa having more similar trophic ecologies, whereas smaller SDNND indicates a more uniform spacing of taxa in the food web space (Abrantes *et al*., 2014). Thus, smaller values of MNND and SDNND represent greater trophic redundancy, as species have more similar trophic niches. In communities with similar MNND (mean distance) values, smaller SDNND represent higher trophic redundancy. A Bayesian approach to these metrics was performed with the SIAR package in R (Stable Isotope Analysis in R; Jackson *et al*., 2011; Parnell and Jackson, 2008), which allows comparing communities containing different sample sizes. The method also allows propagating sample error on the estimates of the means of community components to provide measures of uncertainty surrounding the metrics, making possible robust statistical comparisons among communities. Standard ellipse areas (SEA) were also calculated with the SIBER package (Stable Isotope Bayesian Ellipses in R; Jackson *et al*., 2011) to quantify the isotopic niche of each community. This Bayesian standard ellipse is less sensitive to low sample size and extreme values than the total area proposed by Layman *et al*. (2007b). Therefore, it is a more robust approach for comparisons between communities.

Statistical analyses

All statistical analyses were performed using R software, version 3.6.0. (R Core Team, 2019). We used Linear Models in this study as four values in the covariate (pollution) seem to be not enough for reliable discrimination between linear and non-linear curve fitting (Jenkins and Quintana-Ascencio, 2020). For $\delta^{13}C$ and $\delta^{15}N$ of each resource and taxon, biofilm stock and fine detritus Linear Models were built by including TDN (covariate), Reach (factor) and their interaction as sources of variation. The same sources of variation were used for coarse detritus stock in Linear-Mixed Effects Models (function lme, in R package nlme (Pinheiro *et al*., 2020)) with Sampling campaign as random factor. Variance components of Mixed Effects Models were estimated by means of restricted maximum likelihood and p values estimated by means of likelihood ratio tests (Pinheiro and Bates, 2006). We also divided samples of each food web in four functional groups: basal resources, primary consumers, predatory invertebrates, and fish. We analysed the stable isotopes of these groups by means of Linear-Mixed Effects Models with Taxa as random factor (except for predatory invertebrates which only contained one taxon and were analysed by means of a Linear Model). FCL was also modelled with Linear Models including TDN, Reach and their interaction as sources of variation. To test for the effect of water diversion, pollution and their interaction on diet contribution analyses and the iso-space metrics, we used Generalised Linear Models (GLMs) on the posterior estimates of the Bayesian models, since including these variables into the Bayesian models caused a lack of convergence. We included 3000 posterior estimates on diet contribution analyses and 4000 posterior estimates in the iso-space metrics analyses for each variable and community. Different numbers of posterior estimates were included in GLMs because settings to avoid convergence problems in Bayesian models differed. Posterior estimates related to diet contribution analyses were adjusted to a binomial distribution (link: logit) and estimates related to community iso-space metrics followed Gaussian distribution (link: identity) (Zuur *et al*., 2009). Different GLMs were built for each variable using TDN, Reach and their interaction as sources of variation: null model, two models with a single source of variation, a model with both sources of variation and the maximal model, which also included the interaction term. As the sample size was large, Bayesian Information Criterion (BIC) was used to penalize size and select the best explanatory model in each case (Brewer *et al*., 2016). Model selection was made with the 'modelsel' function of the MuMIn package (Barton, 2020). Due to the large amount of posterior estimates, violin plots were used instead of boxplots to show the distribution of the results whenever Bayesian modelling was applied. For all the linear models we repeated the analysis considering River as a factor instead of TDN as covariate and we tested for pairwise differences between reaches of the same river by means of Post Hoc analysis using the t-statistic (Multcomp package, Hothorn *et al*., 2008).

Results

How did water diversion affect food web complexity?

Water diversion had important effects at the base of the food web that did not propagate to higher trophic levels. This stressor reduced coarse detritus stock by 17.6 % on average from control to diverted river sections ($F_{1,208} = 17.72$, $p < 0.001$, coefficient Reach Diverted = -0.28; Fig. 2a, Table S4), although no within-river differences were observed in the pairwise comparisons (Fig. 2a). Fine detritus showed an overall increase downstream from the weirs ($F_{1,140} = 5.23$, $p = 0.024$, coefficient Reach-Diverted = 0.16; Fig. 2b, Table S4), which was driven by the significant difference between reaches of the most polluted river (Fig. 2b). Biofilm biomass, however, was unaffected by diversion ($F_{1,140} = 0.10$, $p = 0.752$, Fig. 2c, Table S4). The contribution of different basal resources to the diet of primary consumers showed different patterns. Coarse detritus (alder) contribution showed a slight decrease on diverted reaches (Table 3; Fig. 3a; Table S5), which was mainly driven by the pairwise difference between reaches of one of the rivers (Fig. 3a). Similarly, the overall contribution of autochthonous resources also decreased on diverted reaches (Table 3; Fig. 3c; Table S5). Contrarily, fine detritus contribution increased on diverted sites (Table 3; Fig. 3b; Table S5). The $\delta^{15}N$ signatures showed no difference between control and diverted reaches, neither for the entire community nor for functional groups (Fig. 4a, Fig. S2; Table S6) or most of the analysed taxa (Table S7). Consequently, we found similar maximum food chain length (FCL) in diverted and control reaches ($F_{1,35} = 2.35$, $p = 0.134$, Table S3, Fig. 5). In addition, the same fish species were found in both reaches, except for Kadagua that only shared 3 out of the 7 species present in the river (Table S3). Other aspects of trophic structure were weakly affected by water diversion, with a small increase in trophic diversity (CD) and trophic redundancy (lower MNND; although SDNND was higher, and represented more heterogeneous spacing of taxa), and a slight decrease in community niche space (SEA) (Table 4).

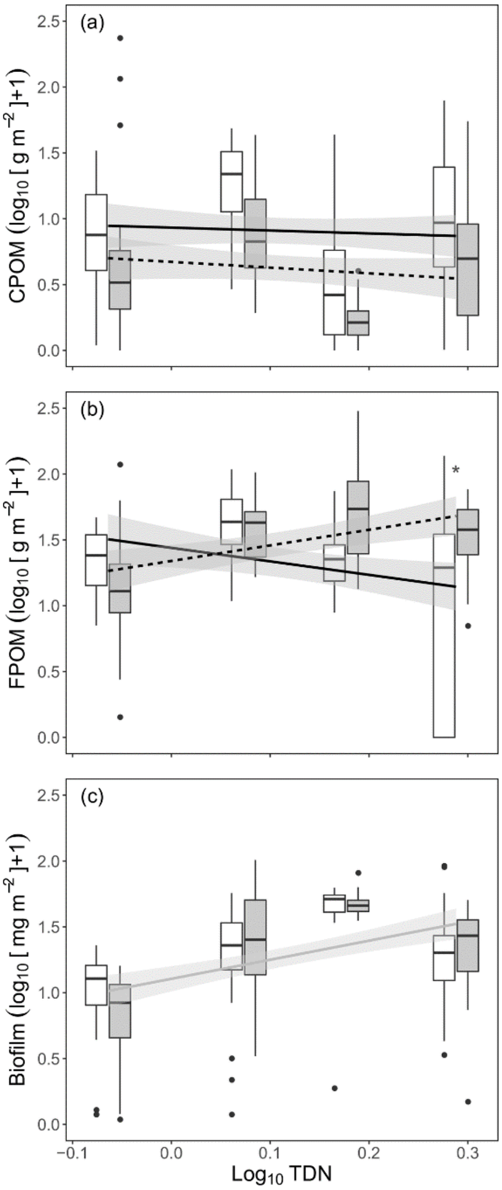


Fig. 2. Resource abundance in the studied reaches (white for control; grey for diverted): (a) coarse detritus (CPOM), (b) fine detritus (FPOM) and (c) biofilm represented along the total dissolved nitrogen (TDN) gradient. The box plots show the median, the interquartile range and the tails of the distribution, and dots represent outliers. Regression lines are drawn with the significant coefficients from linear (biofilm and fine detritus) and linear mixed effect models (coarse detritus). A single grey line is shown when only the TDN was significant in the model, and black regression lines (solid line for control; dashed line for diverted) are drawn when the effect of the diversion differed. Bands around the line represent the 95% confidence interval. Significant differences between the control and diverted reaches within each river are marked with an asterisk.

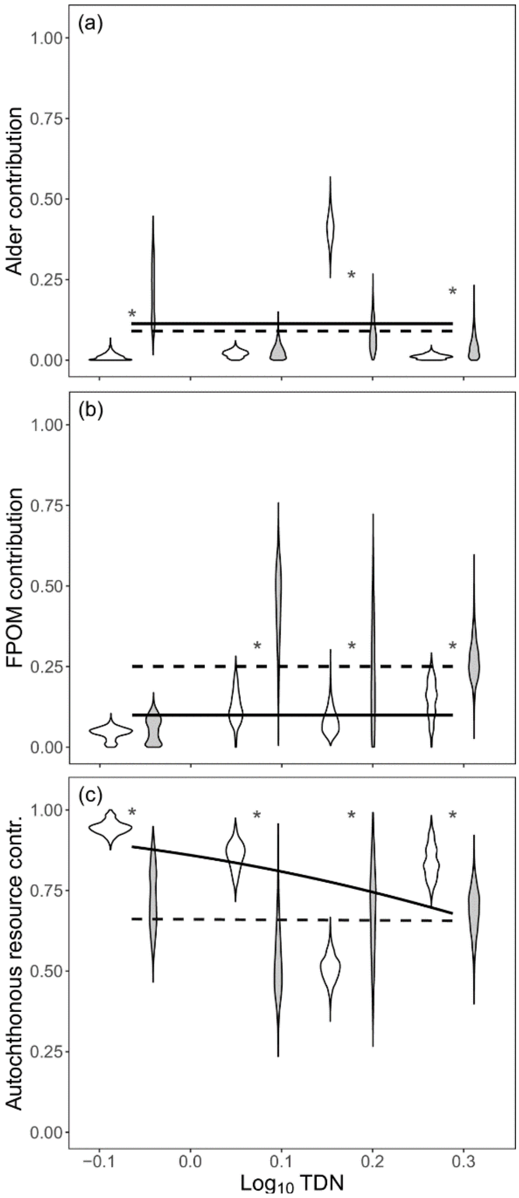


Fig. 3. Bayesian posterior estimates showing the contribution of (a) Alder, (b) fine detritus (FPOM) and (c) autochthonous resources (biofilm, filamentous green algae, bryophytes and macrophytes) to the diets of consumers (white for control; grey for diverted) along the total dissolved nitrogen (TDN) gradient. Black regression lines (solid line for control; dashed line for diverted) are drawn according to the preferred model. Significant differences between the control and diverted reaches within each river are marked with an asterisk.

Table 3. Model-selection for the drivers (pollution –Log$_{10}$TDN-, water diversion -Reach-, both -Log$_{10}$TDN + Reach- and their interaction -Log$_{10}$TDN *Reach-) affecting basal resource (alder, fine detritus and autochthonous resources) contribution to primary consumers. Degrees of freedom (df), log-likelihood ratios (logLik), Bayesian Information Criterion (BIC), and the difference with the model with lowest value (ΔBIC) are given. Models with the lowest BIC are shown in bold. Coefficients for the best model are shown.

Resource consumption	Model	df	logLik	BIC	ΔBIC	Coefficients		
						Log$_{10}$TDN	Reach (D)	Log$_{10}$TDN : Reach (D)
Alder	**Reach**	**2**	**-2739.7**	**5499.6**	**0**		**-0.245**	
	Null	1	-2746.6	5503.3	3.68			
	Log$_{10}$TDN + Reach	3	-2742.1	5514.5	14.85			
	Log$_{10}$TDN	2	-2749.0	5518.1	18.51			
	Log$_{10}$TDN * Reach	4	-2768.8	5577.9	78.30			
Fine detritus	**Reach**	**2**	**-6067.3**	**12154.8**	**0**		**1.107**	
	Log$_{10}$TDN * Reach	4	-6228.3	12497.0	342.12			
	Log$_{10}$TDN + Reach	3	-6241.3	12512.8	357.99			
	Null	1	-6527.1	13064.3	909.50			
	Log$_{10}$TDN	2	-6693.5	13407.2	1252.33			
Autochthonous resources	**Log$_{10}$TDN * Reach**	**4**	**-10473.3**	**20987**	**0**	**-3.692**	**-0.716**	**3.624**
	Null	1	-10584.3	21178.6	191.66			
	Log$_{10}$TDN	2	-10587.4	21195.0	208.02			
	Reach	2	-10598.7	21217.5	230.57			
	Log$_{10}$TDN + Reach	3	-10604.0	21238.2	251.28			

Did moderate levels of pollution increase food web complexity?

Pollution modified the base of the food web and lead to important changes in food web structure. Biofilm biomass increased along the pollution gradient, most clearly at the beginning of the gradient (Table S4, $F_{1,140} = 28.35$, $p < 0.001$, coefficient log$_{10}$TDN = 1.26 Fig. 2c). However, the amounts of coarse and fine detritus showed no significant relationship with the pollution gradient ($F_{1,208} = 1.54$, $p = 0.216$ and $F_{1,140} = 0.08$, $p = 0.771$ respectively, Fig. 2a and b). There was an overall decrease of the contribution of autochthonous resources to the diet of consumers in control reaches (Table 3; Fig. 3c; Table 5S), with no other basal resources changing their overall contribution (Table 3; Fig. 3a and b; Table S5). The δ^{15}N signatures increased significantly with pollution for the entire community, each functional group and most of the analysed taxa (Fig. 4a, Fig. S2; Table S6; Table S7). In addition, the maximum FCL (Fig. 5, Table S3) increased significantly with pollution ($F_{1,35} = 138.06$, $p < 0.001$). Besides differences in the identity of the apex predator among rivers, trophic position of every fish species increased with pollution (Table S3). This stressor also affected every dimension of trophic structure, increasing trophic diversity (CD) and community niche space (SEA) and reducing redundancy (higher MNND; although SDNND was lower, and represented more homogeneous spacing of taxa) (Table 4).

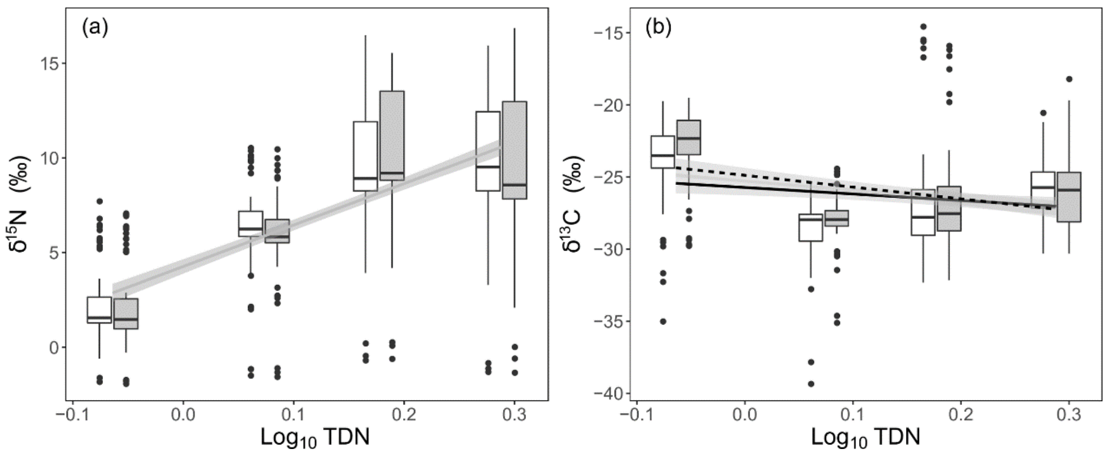


Fig. 4. (a) Nitrogen stable isotope ratios ($\delta^{15}N$, ‰) and (b) Carbon stable isotope ratios ($\delta^{13}C$, ‰) of the entire community in the studied reaches (white for control; grey for diverted) represented along the total dissolved nitrogen (TDN) gradient. The box plots show the median, the interquartile range, and the tails of the distribution, and dots represent outliers. A single grey regression line is represented when only the TDN gradient was significant and black regression lines (solid line for control; dashed line for diverted) are drawn when the effect of the diversion differed. Bands around the line represent the 95 % confidence interval.

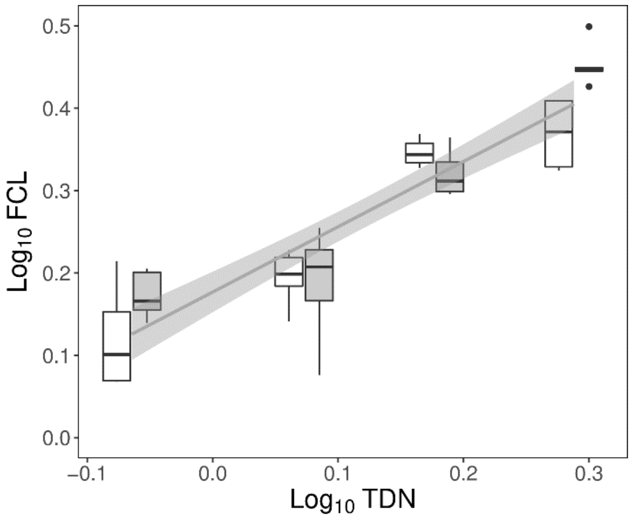


Fig. 5. Maximum food chain length (FCL) in the studied reaches (white for control; grey for diverted) represented along the total dissolved nitrogen (TDN) gradient. The box plots show the median, the interquartile range and the tails of the distribution. A single grey regression line was represented as only the TDN gradient was significant. Bands around the line represent the 95 % confidence interval.

Table 4. Model-selection for the drivers (pollution -Log$_{10}$TDN -, water diversion -Reach-, both -Log$_{10}$TDN + Reach- and their interaction -Log$_{10}$TDN : Reach-) affecting community iso-space metrics of the consumers (invertebrates and fish). Degrees of freedom (df), logarithmic transformation of the likelihood function used to fit models (logLik), Bayesian information criterion (BIC), and difference between a given model and the model with the lowest BIC value (ΔBIC) are given. The most parsimonious model for each community metric is shown in bold. Described community metrics are: CD (distance to centroid representing trophic diversity), SEA (standard ellipse area regarding the niche space), MNND (mean nearest neighbor distance, related to trophic redundancy), and SDNND (standard deviation of the nearest neighbor distance also related to trophic redundancy).

Community metrics	Model	df	logLik	BIC	ΔBIC	Coefficients		
						Log$_{10}$TDN	Reach (D)	Log$_{10}$TDN : Reach (D)
CD	**Log$_{10}$TDN * Reach**	**5**	**-4064.04**	**8180.0**	**0**	**0.270**	**0.034**	**1.167**
	Log$_{10}$TDN + Reach	4	-5241.88	10525.3	2345.30			
	Log$_{10}$TDN	3	-5298.68	10628.5	2448.52			
	Reach	3	-7503.20	15037.5	6857.56			
	Null	2	-7552.52	15125.8	6945.84			
SEA	**Log$_{10}$TDN * Reach**	**5**	**-76361.37**	**152774.6**	**0**	**13.423**	**-2.075**	**-4.480**
	Log$_{10}$TDN + Reach	4	-76556.47	153154.4	379.83			
	Log$_{10}$TDN	3	-78842.71	157716.5	4941.95			
	Reach	3	-80779.97	161591.1	8816.46			
	Null	2	-82564.15	165149.1	12374.46			
MNND	**Log$_{10}$TDN * Reach**	**5**	**-7159.00**	**14369.9**	**0**	**2.124**	**-0.015**	**0.620**
	Log$_{10}$TDN + Reach	4	-7440.63	14922.8	552.89			
	Log$_{10}$TDN	3	-7450.10	14931.3	561.44			
	Null	2	-19126.94	38274.6	23904.75			
	Reach	3	-19122.37	38275.9	23906.00			
SDNND	**Log$_{10}$TDN * Reach**	**5**	**-29462.16**	**58976.2**	**0**	**-3.293**	**0.129**	**2.039**
	Log$_{10}$TDN + Reach	4	-30207.15	60455.8	1479.60			
	Log$_{10}$TDN	3	-30378.40	60787.9	1811.73			
	Reach	3	-33470.14	66971.4	7995.21			
	Null	2	-33609.93	67240.6	8264.43			

Did food web complexity increase even further when water pollution and diversion interacted?

With the increasing pollution, fine detritus abundance in the diverted reaches surpassed the abundance found in control reaches (interaction: $F_{1,140} = 16.32$, $p < 0.001$, Fig. 2b, Table S4), with the largest, and significant, difference observed in the most polluted river. However, the stock of coarse detritus and biofilm showed no significant interaction between stressors ($F_{1,208} = 0.17$, $p = 0.684$ and $F_{1,140} = 0.47$, $p = 0.493$, Fig. 2a and c). Only contribution of autochthonous resources showed an interaction between pollution and water diversion, which decreased in the control reaches along the pollution gradient but remained constant in the diverted ones (Table 3; Fig. 3c; Table S5). Neither δ^{15}N signatures (Fig. 4a, Fig. S2; Table S6) nor maximum FCL ($F_{1,35} = 0.15$, $p = 0.702$, Table S3, Fig. 5) responded interactively to both stressors. However, δ^{13}C values for the entire community and for fish decreased more along the pollution gradient in diverted than in control reaches (Fig. 4b; Table S6). Community-wide metrics of consumers were also best modelled by considering the interaction term (Table 4). Trophic diversity (CD, Fig. 6a; Table S8) became larger in the diverted reaches of the most polluted rivers (Table 4). These rivers also showed larger community niche space (larger SEA, Fig. 6b; Table S8) differences between control and diverted reaches, with the smallest difference between reaches in the least polluted river (Table 4). Trophic redundancy decreased with pollution (higher MNND, Fig. 6c; Table S8), with larger reductions (steeper positive slope) for diverted sites (Table 4). Evenness of this metric (SDNND) was lower in the diverted site of the less polluted river, but higher in the diverted sites of the other three rivers (Fig. 6d; Table S8).

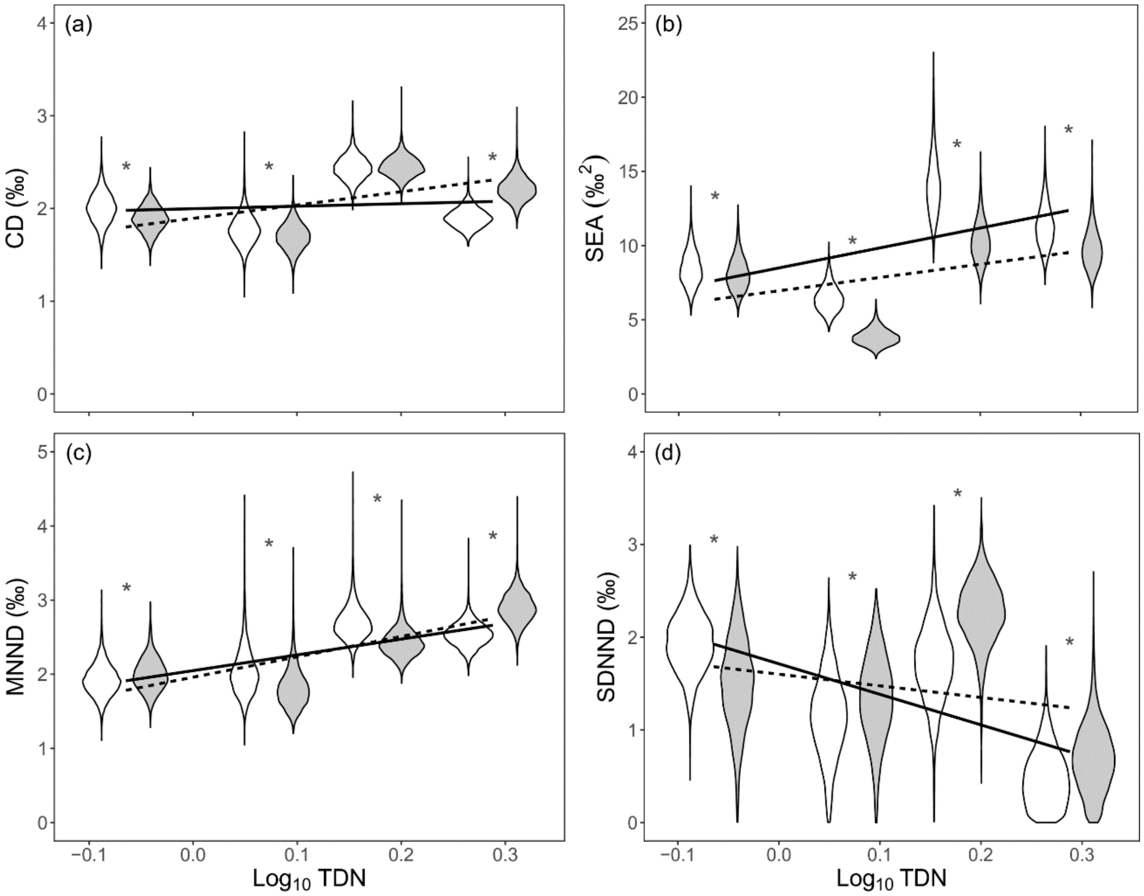


Fig. 6. Bayesian posterior estimates of community wide metrics of the iso-space for consumers: (a) distance to centroid (CD), (b) standard ellipses area (SEA), (c) mean nearest neighbour distance (MNND) and (d) standard deviation of nearest neighbour distance (SDNND). These metrics provide information about trophic diversity, community niche space and redundancy of the eight consumer communities (white for control; grey for diverted) along the total dissolved nitrogen (TDN) gradient, respectively. Black regression lines (solid line for control; dashed line for diverted) are drawn according to the preferred model. Significant differences between the control and diverted reaches within each river are marked with an asterisk.

Discussion

Water diversion and pollution are two pervasive stressors affecting freshwater ecosystems (Dudgeon *et al.*, 2006). Our study has identified the unique and joint effects of both stressors on different dimensions of the complexity of freshwater food webs. We found that water diversion modified the base of the brown food web by reducing the abundance of detritus. In contrast, nutrients from polluted water stimulated the base of the green food web by promoting biofilm production. How these changes at the base of the food web propagated to higher trophic levels differed between stressors. Water diversion had little effect on the structure of the entire food web but nutrient pollution increased its complexity. Interactive, although weak, effects were very common among the response variables.

The effects of water diversion at the base of the food web did not propagate to higher trophic levels

The reduction of stocks of detritus in the diverted reaches agrees with previous studies (Casas *et al.*, 2000; Martínez *et al.*, 2013). This effect is likely to be a consequence of the retention of detritus in the impoundments above weirs (Schmutz and Moog, 2018), and its deviation through the diversion canals (Arroita *et al.*, 2015). These impoundments reduce the size of coarse detritus very efficiently and export fine detritus downstream (Mbaka and Wanjiru Mwaniki, 2015). Concerning autotrophic basal resources, biofilm can respond non-linearly to river flow and water velocity. Water velocity increases nutrient exchange rates, enabling faster biofilm growth (Dewson *et al.*, 2007), with the highest shear forces limiting biofilm accrual (Hondzo and Wang, 2002). Nevertheless, water diversion did not significantly change biofilm biomass in our study. The few instant velocity measures we had available, although not enough to properly capture flow velocity differences among reaches, did not suggest big velocity changes from control to diverted reaches, in accordance with the lack of changes in biofilm biomass.

Supporting our first hypothesis, the reduction of detritus by water diversion was followed by lower contribution to consumers' diets. However, autochthonous resources remained the main contributors to the diet of primary consumers, even in the diverted reaches, where their contribution was lower than in the control reaches. Considering that water diversion did not change the stock of biofilm, the reduction in autochthonous resource contribution in diverted reaches suggests that the turnover of the biofilm was reduced with the diversion. However, biofilm was not the only autochthonous resource sampled for SIA, and thus, we cannot determine whether turnover or a change in the stock of other autochthonous resources of low abundance was responsible for this decrease. The reduced contributions of coarse detritus and autochthonous resources to the diets in the diverted sites were compensated by an increase in the contribution of fine detritus, which can be linked to the larger abundance of this resource in the diverted sections.

Many studies suggest complex relationships between FCL and ecological drivers, such as ecosystem size, perturbations and resource availability (e. g. Post, 2002b; Takimoto *et al.*, 2012; Takimoto and Post, 2013). In our study, FCL was not reduced because of habitat contraction nor flow reduction driven by water diversion. Neither was it reduced due to the decrease in the stock and contribution of detritus in the diverted sites, which is poorer in nutrients than the autochthonous basal resources (Cross *et al.*, 2005). The ability to incorporate a basal resource into the biomass of consumers is given by its abundance and its biochemical composition (Brett *et al.*, 2017). Algae can support upper trophic levels across many aquatic systems due to the high nutritional quality of their amino and fatty acids (Brett *et al.*, 2017) and can be more relevant than the detrital input in determining food web structure (Townsend *et al*., 1998). Thus, longer food chains can be held in more productive ecosystems (Post, 2002b). We did not observe any change in the stock of biofilm due to the diversion; however, the lower contribution of autochthonous resources to the diets of consumers in the diverted reaches compared to control reaches (mainly in the less polluted streams) should also have been accompanied by a reduction of the maximum FCL, which did not happen. Fine detritus, richer in nutrients than coarse detritus (Cross *et al*., 2005), contributed more in the diets of consumers in the diverted sites. The shift from one nutrient-rich resource to another could have maintained the consumer-resource stoichiometric imbalance, and thus, FCL. Kautza and Sullivan (2016) assigned changes in FCL of a regulated river to a combination of mechanisms such as addition and deletion of top predators and insertion of intermediate predators, shifts in the degree of omnivory, and changes in the strength of intraguild predation. Nevertheless, we found the same set of top predators when comparing control and diverted reaches in three out of four rivers, explaining the lack of effects of the diversion on FCL. Similar results to ours were reported by Walters and Post (2008), who did not observe a decrease in FCL as a consequence of water diversion. However, they described a shift in body-size structure, suggesting that the structural complexity of the food web allowed the conservation of the FCL.

We expected diversification of the trophic niches due to the reduction of relative abundance the low-quality coarse detritus in comparison to the abundance of biofilm and fine detritus driven by water diversion. In line with this, Kaymak *et al*. (2018) reported higher trophic diversity and a larger community niche space in the regulated reach downstream from a large dam. They linked it to a higher dominance of trophic generalists, who can shift among alternative resources (Layman *et al*., 2007b). However, the shift they found was related to decreased fish diversity in the downstream reach, which has not been corroborated in our study. The changes we observed in the iso-space metrics occurred regardless of the lack of large taxonomic changes, at least for top predators. In addition, our models indicate a larger trophic diversity and a smaller community niche space in the diverted reaches for all our systems when looking at the selected consumer taxa. Thus, water diversion produced a collapse of generalist species in the iso-space (i.e., the core of the iso-space). In contrast, more specialised consumers and/or species at the top and bottom of the food webs expanded the overall isotopic space of the community. A plausible explanation for this contrasting pattern can be found in the competition exerted by the more specialised consumers at the edges of the iso-space that might have reduced resource availability for the consumers at the core of the food web.

Pollution promoted biofilm production and increased food web complexity

A non-linear effect of nutrient pollution on biofilm production is frequently described in the literature, with stimulating effects of moderate levels of nutrients (e.g. Ardón *et al*., 2021; Pereda *et al*., 2020; Ribot *et al*., 2015), where subsidy effects of nutrients override toxic effects of other compounds. Similarly, along our pollution gradient, biofilm biomass increased. In addition, previous studies that assessed the effects of land use (and thus, of nutrient concentrations; Baumgartner and Robinson, 2017; Pastor *et al*., 2014; Price *et al*., 2019) stated that all community compartments increased in δ^{15}N along the pollution gradient; a result that was completely paralleled in our study. Both total nitrogen and δ^{15}N concentration can be associated with agriculture runoff (Bergfur *et al*., 2009; Harrington *et al*., 1998; Pastor *et al*., 2014) and urbanisation (Pastor *et al*., 2014; Smucker *et al*., 2018), and thus, are often correlated. Additionally, our study highlights that the δ^{15}N increase along the TDN gradient was stronger for consumers than for basal resources, which points towards a reorganization of food webs, and not only to a propagation of the isotopic signal of basal resources. This reorganization was also represented by the longer FCL with the increase in nitrogen, which is in line with previous observations (Kaunzinger and Morin, 1998) and in accordance to the productivity hypothesis (Pimm, 1982). Although other variables, such as water temperature and conductivity, covaried with the pollution gradient, the good fit between $\delta^{15}N$ or FCL with water TDN points to pollution, and specially nutrient concentration, as the main driver shaping food webs in this study.

With more nutrients in the water column and higher biofilm biomass, a scarce but high-quality resource, we anticipated a reorganization of the niche space of the consumers, with more separate and diverse niches. Moderate nutrient pollution, and consequent biofilm availability, have been linked to a larger isotopic variability (García *et al*., 2017; Parreira de Castro *et al*., 2016) as isotopic diversity of basal resources increases. However, further pollution of water by nutrients can also cause a drastic reduction of the diversity of resources (reducing isotopic variability among the available resources), leading to narrower isotopic variation among consumers (García *et al*., 2017). In our study, we observed an overall increase in trophic diversity and a decrease in redundancy along the pollution gradient (i.e. a diversification on consumers' diet and a more uniform spacing of taxa), suggesting that our systems did not suffer from the effects of severe nutrient pollution. However, we must bear in mind that the works cited above are based on isotopic analyses of the entire community, whereas we have centred our study on the core communities of the studied systems, i.e. the same six invertebrate taxa that appeared in all sampling sites plus fishes. Thus, despite responses in our study cannot be attributed to interspecific, but to intraspecific variation, the isotopic patterns are similar.

Effects of water diversion and pollution on food web complexity were exacerbated in combination

When dealing with multiple stressors in the same study it is of interest to rank the stressors in order of ecological relevance and to describe the kind of interaction that they create in response variables. Few studies have already addressed the combined effects of flow reduction and nutrient enrichment (e.g. Elbrecht *et al*., 2016; Lange *et al*., 2014; Matthaei *et al*., 2010). In mesocosms studies, Elbrecht *et al*. (2016) and Matthaei *et al*. (2010), observed pervasive and stronger effects of flow reduction than those associated with nutrient enrichment on the studied variables regarding benthic macroinvertebrates and algal biomass. In contrast, in a field study, Lange *et al*. (2014) showed that nutrient pollution had a larger effect than water diversion on fish populations. The severity of both stressors can vary hugely, as droughts can be created by diversion, and some local extinctions can be the outcome of nutrient pollution. In our case, food webs were more sensitive to pollution than to water diversion. Moreover, pollution was modulating the response of some food web properties to diversion. For instance, when focusing on the iso-space metrics of the selected consumer taxa of the studied rivers, interaction was the norm: trophic diversity was higher and redundancy lower in the diverted sites of the most polluted rivers. Thus, the increase of the availability of biofilm along the pollution gradient led detritus-deprived consumers of the diverted reaches to expand further their diet towards autotrophic resources.

Conclusions

Ecosystems face multiple stressors. It is crucial to study multiple stressors simultaneously to understand interactive effects, create predictive tools and rank their relevance based on their relative ecological impact. In this study based on a representative subset, both pollution and water diversion affected food webs by means of bottom-up mechanisms and nutrient pollution intensified the effects of diversion on the community food web structure. We expect these results to be widespread and strong across freshwater ecosystems that depend mostly on low quality detrital resources, whereas we anticipate these effects to be weaker in systems that mostly depend on high-quality resources, such as biofilm. In addition, both pollution and water diversion can vary in their intensity, resulting in different outcomes on food webs. More severe degrees of pollution and larger water removals are expected to trigger local extinctions and hence food web simplification. It is uncertain to what extent non-linear responses to both stressors will emerge as their intensity increase (Hillebrand *et al*., 2020). We believe our results will foster further research on the interactive effects of multiple stressors of varying intensities to better understand their effects on freshwater ecosystems globally.

Acknowledgements

This research was supported by the Spanish Department of Economy, Industry and Competitiveness through the project GL2016-77487-R (DIVERSION), the European Social Fund, the Basque Government (Consolidated Research Group IT951-16) and the Biscay Province Council (61/2015). Authors also acknowledge the financial support from pre-doctoral fellowship from the Basque Government (I. de Guzman), pre-doctoral fellowship from the Spanish ministry (A.V. Perez-Calpe) and pre-doctoral fellowship from CONICET (P. Altieri). D. von Schiller is a Serra Húnter Fellow. J. M. Montoya is funded by the FRAGCLIM Consolidator Grant (number 726176) from the European Research Council under the European Union’s Horizon 2020 Research and Innovation Program and by the "Laboratoires d’Excellences (LABEX)" TULIP (ANR-10-LABX-41). Authors are especially grateful to Janire Diez for her assistance in laboratory analyses, to EKOLUR (Asesoría ambiental-Ingurumen aholkularitza), SGIker technical and human support (UPV/EHU, MICINN, GV/EJ, ESF) and UC Davis Stable Isotope Facility. Comments from the anonymous reviewers significantly improved the manuscript.

Conflict of interest

Authors declare that there is no conflict of interest regarding the material discussed in the manuscript.

Data availability statement

The original data that support the findings of this study are openly available in Figshare and GitHub (https://doi.org/10.6084/m9.figshare.17014022.v1).

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Chapter 2

Pollution modulates the effects of water diversion on stream food web energy fluxes

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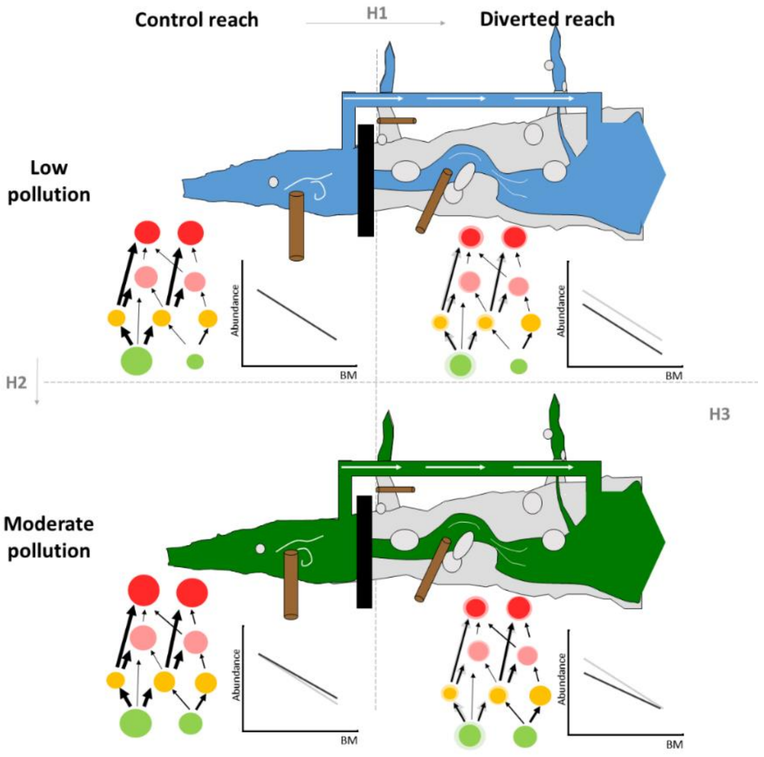
Abstract

Water diversion and pollution accentuated by the increasing human population are two pervasive stressors for river ecosystems that often arise concurrently. Although individual effects of both stressors on stream communities are quite well described, still how the interactive effects between water diversion and pollution affect communities and energy transfer across food webs remains unknown. We hypothesised that with the interaction between both stressors, invertebrate abundance and diversity would decrease and that total energy fluxes would diminish due to the stronger reduction in detritivory caused by water diversion than the increase of herbivory promoted by pollution. We also expected a shift in size spectra with increased energy transfer between trophic levels along the pollution gradient which would support larger individuals, but an overall decrease in abundance due to water diversion. To test these hypotheses, we selected four rivers in a range of pollution subject to similar water diversion schemes and compared food webs upstream and downstream of their diversion weirs. Both stressors changed the availability of basal food resources, with water diversion affecting brown food web by decreasing stocks of detritus and pollution promoting the green food web by enhancing biofilm stocks. The propagation of the effects to higher trophic levels differed with each stressor. Water diversion for instance, increased community heterogeneity, whereas pollution decreased it. Moreover, diversion reduced the total energy fluxes through a decrease in detritivory, whereas pollution only induced changes within omnivores, increasing herbivory, carnivory and dependency on biofilm. Notwithstanding, interactive effects of both stressors were common among most of the response variables. Thus, although most of the variables of interest seemed to be more sensitive to water diversion, pollution often modulated their response. Our study highlights the importance of assessing the effects of multiple stressors to properly understand their interactive effects and classify their relevance according to their impact in ecosystems for a proper management of ecosystems.

Key words: *Freshwater food web, water diversion, pollution, community size-spectra, diversity, energy fluxes*



Introduction

The increase of human population and the intensification of their activities have raised water demand (Crist *et al*., 2017; Ripple *et al*., 2017). In Europe, for instance, the impact of human activities on rivers is severe (Tockner *et al*., 2009), as in order to satisfy the demand of water for agriculture, industry and domestic use (Albert *et al*., 2021), they are increasingly being regulated by barriers, being weirs and dams the most frequently built structures (30.5% and 9,8%, respectively) to control and divert water flows (Belletti *et al*., 2020). These structures have multiple effects on ecosystems. The barriers themselves disturb the dispersion of aquatic organisms by disrupting connectivity across the fluvial network (Brooks *et al*., 2018; Jones *et al*., 2020), which affects community structure (Carpenter-Bundhoo *et al*., 2020; Munasinghe *et al*., 2021). Additionally, water diversion driven by these infrastructures can modify environmental conditions through narrowing wetted channel, reducing flow velocity and water depth and altering water physico-chemistry (Zoë S Dewson *et al*., 2007), which leads to changes in community composition (González and Elosegi, 2021; Munasinghe *et al*., 2021). Water diversion can also alter basal resource availability (Power *et al*., 2013), for instance reducing the stock of coarse detritus downstream from this structures as it can be retained in the impoundments (Schmutz and Moog, 2018) and transported through diversion canals (Arroita *et al*., 2015). This might have special importance in forested low order streams, where detritus is the main energy source (Vannote *et al*., 1980; Zhang *et al*., 2019) since primary production is usually limited by nutrients (Elser *et al*., 2007; Tank and Dodds, 2003) and canopy cover (Bernhardt *et al*., 2018). Thus, here, primary consumers mainly ingest organic matter inputs colonized by microbes to obtain energy and nutrients (Marks, 2019), strengthening the brown over the green food web.

River ecosystems usually face multiple stressors simultaneously (Ormerod *et al*., 2010; Sabater *et al*., 2018), which may interact in unpredictable ways (Crain *et al*., 2008; Jackson *et al*., 2016; Orr *et al*., 2020) amplifying or lessening the effect of a single stressor depending on the interaction strength and the direction of the interaction (Piggott *et al*., 2015). One of the most pervasive stressor for freshwater ecosystems (Malmqvist and Rundle, 2002; Reid *et al*., 2019) that acts interactively with other perturbations is pollution (Dolédec *et al*., 2021), which degrades water quality and ecosystem status. Chemical pollution constitutes a complex mixture of contaminants and might have contrasting effects on ecosystems depending on the composition of pollutants (Flores *et al*., 2014; Guasch *et al*., 2003), the level of dilution in the receiving water bodies (Carey and Migliaccio, 2009a) and the target organisms (Artigas *et al*., 2014). In addition, depending on their individual effects on biota, some compounds are simply toxic (Patel *et al*., 2020; Vasilachi *et al*., 2021), whereas others, such as nutrients, subsidize biological activity, although they can also become hazardous above a certain concentration (Carey and Migliaccio, 2009b; Wang *et al*., 2019). Moderate concentrations of these last compounds without reaching to harmful levels, reduce nutrient limitations for algal communities (Marcarelli *et al*., 2009) and lead to an increase of their biomass (Keck and Lepori, 2012). In addition, recent studies have emphasised the key role that autotrophic producers, higher-quality food resources than detritus (Cross *et al*., 2005), play in freshwater ecosystems (Brett *et al*., 2017). Even at low abundances they can shift the relevance of green and brown food webs (Crenier *et al*., 2017; Marcarelli *et al*., 2011), in the later one through the stimulation or inhibition of organic matter processing by the biofilm (Halvorson *et al*., 2019, 2016).

These alterations at the base of the food web modify entire assemblages through bottom-up mechanisms (Wallace *et al.*, 1997) as energy and matter pathways are changed. These transformations in composition and structure of communities driven by water diversion and pollution can disturb food webs and end up affecting ecosystem functioning (Thompson *et al.*, 2012; Woodward *et al.*, 2005). Body size is a recognized driver of the position organisms occupy in food webs (Woodward *et al.*, 2005) and the individual body size distribution of the individuals forming the community points out the way in which energy is distributed along the food web (White *et al.*, 2007), since the slope of the relationship between abundance and body mass at log scale informs about the trophic transfer efficiency in the community (Trebilco *et al.*, 2013). Here, a swallower slope indicates that energy is more efficiently transported towards higher trophic levels of the food web, and thus supports higher abundances of the largest individuals (Woodward *et al.*, 2005). Energy transfer efficiency can be altered by modifications in nutrient and resource cycles. Nutrient inputs, for instance, can modify the stoichiometric gap between resources and their consumers, thus affecting trophic transfer efficiency (Mulder and Elser, 2009; Ott *et al.*, 2014; Xu *et al.*, 2015). Slopes of size spectra can also be altered by changes in organisms abundances such as a reduction in the abundance of an specific size class due to toxic compounds (Baho *et al.*, 2019), increases of pollution tolerant taxa (Peralta-Maraver *et al.*, 2019) or decreases in abundances of large body-sized organisms due to water abstraction (Boddy *et al.*, 2020). Thus, ecosystem disturbances can produce changes in the size spectrum of communities and alter their energy fluxes, finally affecting ecosystem functioning (Brose *et al.*, 2017; Mulder *et al.*, 2008).

Isolated effects of water diversion and pollution on food web structure have been previously documented (e.g. Boddy *et al.*, 2020; González and Elosegi, 2021; Haxton and Findlay, 2008; Mor *et al.*, 2019). As far as we know, just a few studies have even assessed the joint effects of nutrient pollution and water diversion by low weirs (de Guzman *et al.*, 2021; Lange *et al.*, 2014) or flow reduction (e.g. Elbrecht *et al.*, 2016; Matthaei *et al.*, 2010) on river biota, structure of food webs and functioning. However, there is still a lack of studies assessing the effects of these two stressors on the organization of food webs and in the efficiency of energy transfer. The aim of our study is to assess the isolated and interactive effects of water diversion and water pollution on the structure of communities, and on the efficiency of energy fluxes across food webs. We hypothesise that (Fig. 1):

On the one hand, dams will act as barriers reducing the amount of coarse detritus reaching downstream. Invertebrate density, and alpha- and beta diversity will decrease downstream from dams. The decrease in coarse detritus will lead to a decrease in detritivory, which will result in a general decrease in energy fluxes of food webs. Generally, energy transfer efficiencies will remain the same, with size spectra showing similar slopes upstream and downstream from dams, however, changes in the intercept will be observed as invertebrate abundance will decrease.

On the other hand, moderate pollution will subsidize biofilm production and affect pollution-sensitive taxa by decreasing alpha- diversity but increasing invertebrate densities. The increase in biofilm will sustain an increase in herbivory, with a general increase in energy fluxes of food webs. Finally, the increase in nutrients with pollution will increase the efficiency of energy transfer along the food web holding more large individuals and thus leading to shallower slopes in the size spectra.

With the interaction between water diversion and pollution, invertebrate abundance and diversity will decrease. In addition, total energy fluxes will diminish as the decrease in detritivory caused by water diversion will be stronger than the increase of herbivory promoted by pollution. Finally, we expect a shift in size spectra since the energy transfer between trophic levels will increase with pollution supporting larger individuals, but the overall decrease in abundance due to water diversion will reduce the intercept.

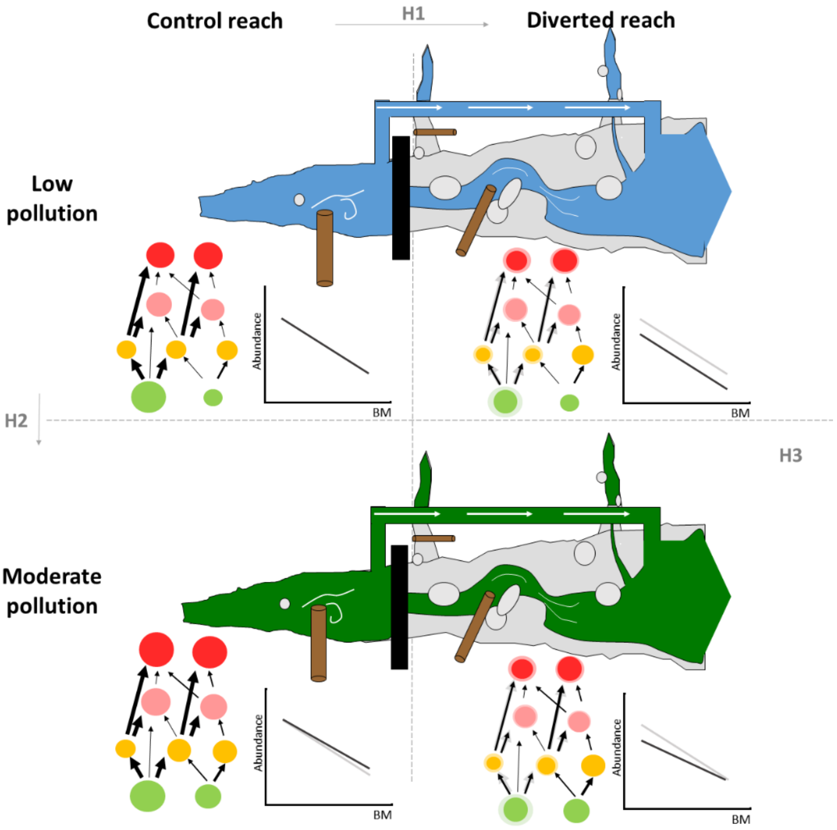


Fig. 1. Conceptual figure of the proposed hypotheses: H1 refers to the hypothesis regarding water diversion, H2 refers to the one related to the increase in pollution and H3 refers to the interaction between the two stressors. Control reach and diverted reach refer to the sampling sites above and below the weirs. Low pollution and moderate pollution is a simplification of the pollution gradient. A simplification of food webs is represented where basal food resources are represented in green, primary consumers in yellow, omnivores in pink and carnivores in red. Arrows between nodes indicate the link and the thickness represents the strength of the relationship. The slope in body mass- abundance size spectra and the nodes of the food web from Control-Low pollution site is redrawn inlighter colors in the other biplots as reference.

Materials and methods

Sampling design and study sites

We selected four rivers within the temperate region of the northern Iberian Peninsula, which differed in their ecological status and water quality (Aguirre *et al*., 2017) (Table 1). The pollution ranged from low to moderate and none of the selected rivers showed a bad ecological status. The cover and maturity of the riparian forests also differed between rivers (higher in Urumea and Leitzaran than in Kadagua and Deba), which was inversely related to the level of urbanisation (Table 1). The four rivers had a similar water diversion scheme, consisting of a low weir (3-6.5 m high) and a canal that can divert up to 90% of the river flow to hydropower. We defined two 100 m-long reaches in each river: a control reach upstream from the stagnant water retained by the weir and a diverted reach in the bypassed section downstream from the weir, but below the direct effect of the water spillage.

Table 1. Main characteristics of the studied rivers. The total annual precipitation and mean annual air temperature are the average values for 2017 and 2018 (www.euskalmet.euskadi.eus). Ecological status and the Referenced Physicochemistry Index (RPI) for the period 2012-2016 are shown (Aguirre *et al*., 2017); the asterisk (*) indicates rivers with a heavily altered hydromorphology. Rivers are ordered following $\log_{10}$(TDN) values from left to right.

		Urumea	Leitzaran	Kadagua	Deba
Basin		Urumea	Oria	Kadagua	Deba
Coordinates of the dam	Latitude	43°12'53.5"N	43°07'57.6"N	43°13'37.9"N	43°09'37.6"N
	Longitude	1°54'16.7"W	1°56'13.4"W	3°00'58.8"W	2°24'08.6"W
Elevation (m asl)		69	354	37	122
Total annual precipitation (mm)		1838.6	2268.4	1288	1316.2
Mean annual air temp. (°C)		13.5	13.6	13.3	12.7
Upstream catchment area (km^2)		186.1	62.8	449	355.1
Land use (%) in upstream catchment area	Urban	0.1	1.1	2.5	4.6
	Agriculture	0.8	10.7	25.8	17.4
	Forestry	98.3	88.2	71.5	77.8
	Water	0.8	0.0	0.3	0.2
Ecological status		Good	Good	Good*	Moderate*
RPI		0.80 ± 0.01	0.79 ± 0.01	0.78 ± 0.01	0.68 ± 0.02
Maximum concession volume (m^3 s^{-1})		5.8	3.0	4.0	5.0

Water and site characteristics

Available information on water characteristics of three sampling campaigns (late spring of 2017, autumn of 2017 and late spring of 2018) were gathered to set the baseline status of each river and include the variability during water diversion and non-diversion periods. Water characteristics did not differ between reaches (Table 2 and S1 from de Guzman *et al*. (2021)), although they differed among rivers. According to the gradient of pollution (represented in our study by the Total Dissolved Nitrogen (TDN) gradient, which ranged from 0.85 ± 0.06 to 1.94 ± 0.35 mg N L^{-1}) Urumea was the least polluted river, followed by Leitzaran, Kadagua and Deba. This gradient correlated with the concentrations of most solutes, pH, conductivity, and temperature (Table S1 from de Guzman *et al*. (2021)) and was related to the Referenced Physicochemistry Index in (Aguirre *et al*., 2017, Table 1).

Sampling and sampling processing

The food-webs of the eight study sites (4 rivers X 2 reaches) were sampled during late spring of 2018. In this period of the year the largest flow differences between upstream and downstream reaches from dams occur because precipitations start to lessen but diversion canals are still active. In summer, further reductions of precipitation and river discharges forces managers to stop abstracting water in order to maintain ecological flows.

Biofilm, benthic organic matter stock and macroinvertebrates

Biofilm biomass was estimated by means of a BenthoTorch fluorometer (Bentho Torch, bbe-Moldaenke, Germany) on 18 cobbles per reach. We collected nine benthic Surber samples (surface of 0.09 m^2, mesh of 0.5 mm) randomly along each reach. For each sample, we gathered the organic matter retained on an 8-mm sieve to obtain the ash free dry mass (by drying at 70 °C – 72h – and ashing the material at 500 °C – 8h–) in each sample. Macroinvertebrates collected in a 0.5-mm sieve were preserved in 96% ethanol. In the laboratory we sorted, identified to the lowest possible taxonomic level following Tachet *et al*., 2010 (mostly to genus-level except for some Diptera identified to subfamily level, Heptageniidae to family level and Annelida to subclass level) and counted them to obtain population densities. In addition, we measured the body length of up to 30 randomly selected individuals of each taxon in every sample (except for oligochaetes, planarians and leeches, which were not measured) with a binocular microscope (Leica M165FC, Wetzlar, Germany) equipped with a Leica DFC310FX camera using “Leica Application suite V4” software program (LAS V4.1). Total body length was considered as the distance from the anterior part of the head to the posterior part of the last abdominal segment excluding antennae and tails (Martínez *et al*., 2016). For gastropod mollusks, we measured the maximum length of the shell (Meyer, 1989), and for crustaceans of the genus *Echinogammarus,* the dorsal length of the first abdominal segment was measured to posteriorly obtain body length (Flores *et al*., 2014). We did not correct length measurements for potential effects of storage in ethanol. We obtained individual body masses (BM) (mg dry weight) using published length-mass relationships (Baumgärtner and Rothhaupt, 2003a; Benke *et al*., 1999; Burgherr and Meyer, 1997; Larrañaga *et al*., 2009; Meyer, 1989; Stoffels *et al*., 2003).

Fish

We conducted fish samplings along the reaches (sampled surface area from 385.3 to 1731.6 m^2) by depletion electrofishing with a backpack-electrofishing unit (Hans Grassl model IG2002/D30). Stop-nets were set at the upstream and downstream ends of the reaches and the required runs were made until the depletion of the captures (Lobón-Cerviá, 1991). All fish were anaesthetized with MS-222, identified, counted and weighed (to the nearest g) (reference number of the ethics commission: M20/2016/135). We converted wet mass into dry mass through conversion factors published in www.fishbase.se.

Invertebrate diversity

We assessed invertebrate taxa diversity through Hill numbers (i.e. number equivalent, ${}^{q}D$ (Jost, 2006)) with the entropart package for R (Marcon and Hérault, 2015). We used Hill numbers of order 0 (^{0}D, species richness, which is insensitive to the abundance of individuals of each taxon, highlighting the response of rare taxa), 1 (^{1}D, the exponential of Shannon's entropy, which weighs each taxon according to its log-transformed abundance), and 2 (^{2}D, inverse of Simpson concentration, which weighs each taxon according to its abundance, highlighting the response of dominant taxa) (Jost, 2006). We computed alpha-diversity per sample in each period and reach for the three Hill number orders and beta-diversity among samples within each period and reach for orders 0 and 1 of diversity measures. Beta-diversity (D_β) for the diversity orders (q) 0 and 1 was transformed from beta entropy (H_β) as described in Marcon and Hérault (2015):

$${}^{q}D_{\beta} = e_q^{\frac{{}^{q}H_{\beta}}{1-(q-1)\,{}^{q}H\alpha}}$$

Food webs, energy flux and dependency on basal resources

We constructed local food webs (9 replicates per river and reach, one per Surber collected) joining information of every resource, benthic invertebrate and fish collected. For every invertebrate taxon in each sample, we calculated mean body mass (BM) from the individual masses measured and estimated the total biomass per node. We also estimated total metabolic rate (MR) for each invertebrate node based on individual MR, calculated for each individual using an allometric equation derived from Gillooly *et al*. (2002):

$$X = \exp((a \cdot \ln(BM) + x_0) + E/kT)\ ,$$

where X is the MR (in watts, W), a is the allometric exponent (0.71), BM is the body mass (g), E is the activation energy (0.63 eV), k is the Boltzmann's constant ($8.62 \cdot 10^{-5}$ eV K^{-1}), T is the temperature (K) and xo is a normalization factor (17.17 for invertebrates and 18.47 for fish). All these parameters were extracted from Brown *et al*. (2004). We gathered mean daily T of the 190 days before the sampling date in each reach. In the case of fish, assuming a homogeneous distribution of fish along the reach we estimated the total biomass in each sample (Surber sampler area, 0.09m^2). In the case of biofilm, we used the average biomass per cobble surface to estimate total biomass in each sample. Fine detritus was a scarce basal resource with a heterogeneous distribution along the reach and was not quantified during the sampling campaign, so it was equalled to the mean biofilm biomass values recorded.

To estimate energy fluxes between nodes of local food webs, we used an adapted food-web energetics approach (Barnes *et al*., 2018; Gauzens *et al*., 2019; Jochum *et al*., 2021) by means of the "fluxweb" package (Gauzens *et al*., 2019). This approach uses allometric scaling laws to quantify MRs (Brown *et al*., 2004). The model assumes a steady-state system, where the energetic losses of nodes in each food web, estimated by MR of consumer j (X_j) and predation on consumer j by higher trophic levels (k), need to be balanced by the energetic gains defined through resource consumption and assimilation (Barnes *et al*., 2018; O'Neill, 1969). The flux of energy $F_{i,j}$ from resource i to consumer j was calculated as

$$\sum_i e_{ij}\, F_{ij} = X_j + \sum_k W_{jk} F_k$$

where e_{ij} is the efficiency in which consumer *j* assimilates the energy consumed from resource *i*. Energy fluxes to each consumer are defined as $F_{ij} = W_{ij}F_j$, where F_j is the sum of all the ingoing fluxes to consumer *j* and W_{ij} is the proportion of F_j obtained from resource/prey *i*, after scaling consumer preferences w_{ij} to the biomass (*B*) of the different resources/preys as:

$$W_{ij} = \frac{w_{ij}B_i}{\sum_k w_{kj}B_k}.$$

For that, an adjacency matrix with possible trophic links among all taxa present in our study and feeding preferences for each possible food resource was created based on the literature (Gray *et al*., 2015; Tachet *et al*., 2010) and our own gut content findings (I. de Guzmán, unpublished data) (See supplementary data set: https://www.dropbox.com/s/l0s0ck2iuqx0x7k/matrix1_Diversion_trophic_links_preferences.csv?dl=0). For carnivore taxa we assumed that preferences were equally distributed amongst prey species. For omnivore invertebrates and primary consumers *w* values were given following preferences in Tachet *et al*. (2010), where traits related to consumed food are quantified using affinity scores between 0 and 5. For omnivores, affinity scores related to predation were equally distributed amongst prey species. For cannibalistic species, we set the preference for cannibalism to 0.01 in the adjacency matrix to minimize the amount of energy a consumer could ingest from its own biomass pool. Assimilation efficiencies ($\mathcal{E}$) for the consumption of food resources were calculated deriving a formula from Lang *et al*. (2017):

$$\mathcal{E} = e^{\mathcal{E}'} \cdot e^{E\frac{T-T0}{kTT0}} / \left(1 + \left(e^{\mathcal{E}'} \cdot e^{E\frac{T-T0}{kTT0}}\right)\right)$$

where $\mathcal{E}'$ is normalization constant for assimilation efficiency (-1.670 for detritivory, 0.179 for herbivory and 2.260 for carnivory), E is the activation energy (0.164 eV), *k* is the Boltzmann's constant and T is the temperature (K). Parameters were extracted from Lang *et al*. (2017).

We calculated whole-food web energy flux as the sum of energy fluxes within each local food web (each Surber sample). To calculate the consumption on biofilm, detritus and preys, we summed all the outgoing energy fluxes from each food resource. Thus, we quantified three consumption pathways: herbivory (consumption of biofilm), detritivory (consumption of coarse and fine detritus) and carnivory (consumption of animals) in the entire food web and within each trophic group (primary consumers, omnivores and carnivores) of each local food web. In addition, we calculated the dependency of each trophic group on basal food resources (biofilm and detritus) with the "NetIndices" package (Soetaert and Kones, 2014).

Body size spectra

We constructed size spectra for the entire community (including both invertebrate and fish assemblages), and for primary consumers, omnivores and carnivores, separately. We used BM of the measured (invertebrates) and weighed (fish) organisms. Since the log-transformed length values followed a normal distribution, we obtained BM of the remaining non-measured invertebrates by means of the "truncnorm" package (Mersmann *et al*., 2018), based on the mean, standard deviation, minimum and maximum values of each taxa in each sample. We used animals with BM higher than 0.1 mg to construct the size spectrum, since organisms with lower weights are assumed to be undersampled as they were mostly, but not always, washed through the 0.5 mm mesh aperture sieves (Gruenert *et al*., 2007). We divided the total range of BM ($\log_{10}$BM) values into 8 logarithmic bins of the same width and regressed density of organisms (N; $\log_{10}$N) against the center of the bin (White *et al*., 2008). The creation of these bins allowed using BM as a covariate in the analyses. The amount of bins influences the estimated regression coefficients, so the same number of bins was used between periods and reaches in each assemblage to allow the comparison of slopes and intercepts.

Statistical analyses

We conducted all the statistical and data analyses using R software, version 3.6.0. (R Core Team, 2019). We performed Linear Models by including Reach as factor, pollution (TDN) as covariate and their interaction as sources of variation. Some variables were log-transformed to fulfil the requirements for Linear Models. We avoided non-linear Models as we considered four values in the covariate (pollution) not to be enough for reliable discrimination between linear and non-linear curve fitting (Jenkins and Quintana-Ascencio, 2020). We repeated all the analysis considering River as factor instead of pollution as covariate to test for pairwise differences between reaches within each river through PostHoc analysis using t-statistic (Multcomp package, Hothorn *et al*. (2008)).

Results

Water diversion reduced the stock of detritus, decreasing detritivory and total energy fluxes but without affecting energy transfer efficiency on food webs

Water diversion had important effects at the base of the food web as the overall amount of coarse detritus stock was reduced in a 26.1 % on average from control to diverted river sections ($F_{1,68}$ = 7.69, p = 0.007; Fig. 2a, Table 2), although no within-river differences were observed in the pairwise comparisons. Biofilm biomass was unaffected by water diversion ($F_{1,140}$ = 0.10, p = 0.747, Fig. 2b, Table 2). In addition, water diversion did not affect the overall alpha diversity regarding taxa richness, Shannon diversity nor Simpson diversity (Fig. 3a, c, e). Beta diversity for taxa richness was higher in diverted reaches than in the control ones ($F_{1,284}$ = 37.05, $p < 0.001$; Fig. 3b, Table 3), evidencing a higher heterogeneity among samples with the diversion due to the larger differences in species richness across communities. Invertebrate density showed a decrease in diverted sites ($F_{1,68}$ = 18.49, $p < 0.001$; Fig. 3f, Table 3). Within-river differences were observed in the pairwise comparisons in the most and least polluted rivers for beta diversity, and in the most polluted river for taxa richness and invertebrate density. Regarding fish assemblages we observed the same fish species in upstream and downstream reaches in each river, but densities in diverted reaches were higher than in control ones in most of the rivers (Table S2). Mean body mass of the three trophic groups did not change with water diversion (Table S1), however, MR of primary consumers was lower in diverted reaches ($F_{1,888}$ = 5.93, p = 0.02; Fig. S1, Table S1), while MR and total biomass of carnivores was larger in these reaches compared to control reaches ($F_{1,328}$ = 9.08, p = 0.003 and $F_{1,328}$ = 6.56, p = 0.011 respectively; Fig. S1, Table S1). Regarding energy fluxes, we observed a decrease in total fluxes with water diversion ($F_{1,68}$ =6.02, p = 0.017; Fig. 4a, Table 4) which was parallel to the decrease observed in detritivory ($F_{1,68}$ =6.86, p = 0.011; Fig. 4c, Table 4). Mostly primary consumers were responsible of the decrease in detritivory from control to diverted reaches as energy fluxes associated to this function significantly diminished within this trophic group ($F_{1,68}$ = 8.7, p = 0.004; Fig. S2, Table S3). We did not observe changes in the dependencies of consumers on basal food resources driven by water diversion (Fig. 5, Table 5).

Finally, size spectra of the entire community did not differ between reaches (Fig. 6a, Table 6), although a decrease in primary consumers' intercept was observed with diversion ($F_{1,48} = 16.71$, p < 0.001; Fig. 6b, Table 6).

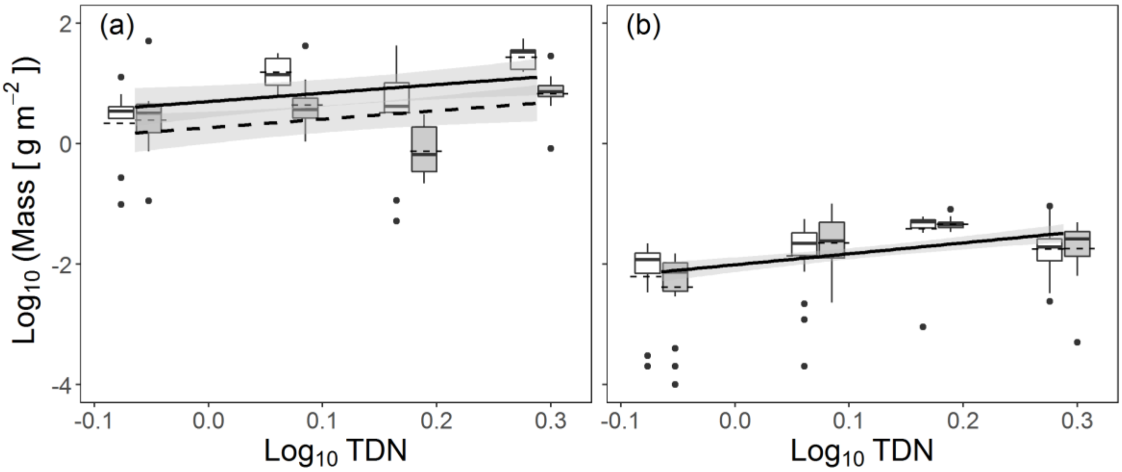


Fig. 2. Resource abundance in the studied reaches (white for control; grey for impact): (a) leaf litter and (b) biofilm represented along the pollution gradient. The box plots show the median, the interquartile range and the tails of the distribution. Dashed lines represent the mean value. A single black regression line is represented when only the TDN gradient was significant and black regression lines (solid line for control; dashed line for diverted) are drawn when the effect of the diversion differed. Bands around the line represent the 95 % confidence interval.

Table 2 Linear model results comparing stock of basal resources between reaches along the pollution gradient. Bold values indicate statistical significance (p < 0.05). Coefficients are shown for significant responses and consider pollution gradient (Log_{10}TDN) and C reaches as reference in all cases.

	Log_{10}TDN			Reach			Log_{10}TDN:Reach	
	F	p	Coeff.	F	p	Coeff.	F	p
Coarse detritus	5.51	**0.022**	2.33	7.69	**0.007**	-0.43	2.36	0.13
Biofilm	26.11	**<0.001**	1.62	0.1	0.747		0.31	0.577

Moderate pollution increased stock of basal food resources, although it was not reflected on the energy transfer of the entire community

Moderate pollution modified the base of the food web by increasing biofilm biomass along the pollution gradient ($F_{1,140} = 26.11$, p < 0.001; Fig. 2b, Table 2), most clearly at the beginning of it. Stock of coarse detritus also increased with pollution, although not as clearly as biofilm ($F_{1,68} = 5.51$, p = 0.022; Fig. 2a, Table 2). Taxa richness, Shannon diversity and Simpson diversity at alpha scale and invertebrate density were not affected by pollution (Fig. 3, Table 3), however, beta diversity for taxa richness and Shannon diversity decreased with it ($F_{1,284} = 23.64$, p < 0.001 and $F_{1,284} = 29.6$, p < 0.001 respectively; Fig. 3b and d, Table 3). Mean body mass of primary consumers and omnivores increased with pollution ($F_{1,888} = 4.35$, p = 0.037 and $F_{1,727} = 5.74$, p = 0.017; Table S1, Fig. S1), as well as MR and biomass of omnivores ($F_{1,727} = 18.73$, p < 0.001 and $F_{1,727} = 13.32$, p

< 0.001) and MR of carnivores ($F_{1,328}$ = 4.7, p = 0.031). Neither total energy fluxes along food webs nor functions varied with pollution (Fig. 4, Table 4), however herbivory and carnivory driven by omnivores increased along the pollution gradient ($F_{1,68}$ = 12.47, p = 0.001 and $F_{1,68}$ = 6.57, p = 0.013; Fig. S2, Table S3). The dependency of this trophic group on biofilm also increased with pollution, leading to a decrease in detritus dependency ($F_{1,68}$ = 5.84, p = 0.018; Table 5, Fig. 5b). Size spectra of the entire community and of each trophic group suffered no variation with pollution (Table 6, Fig. 6).

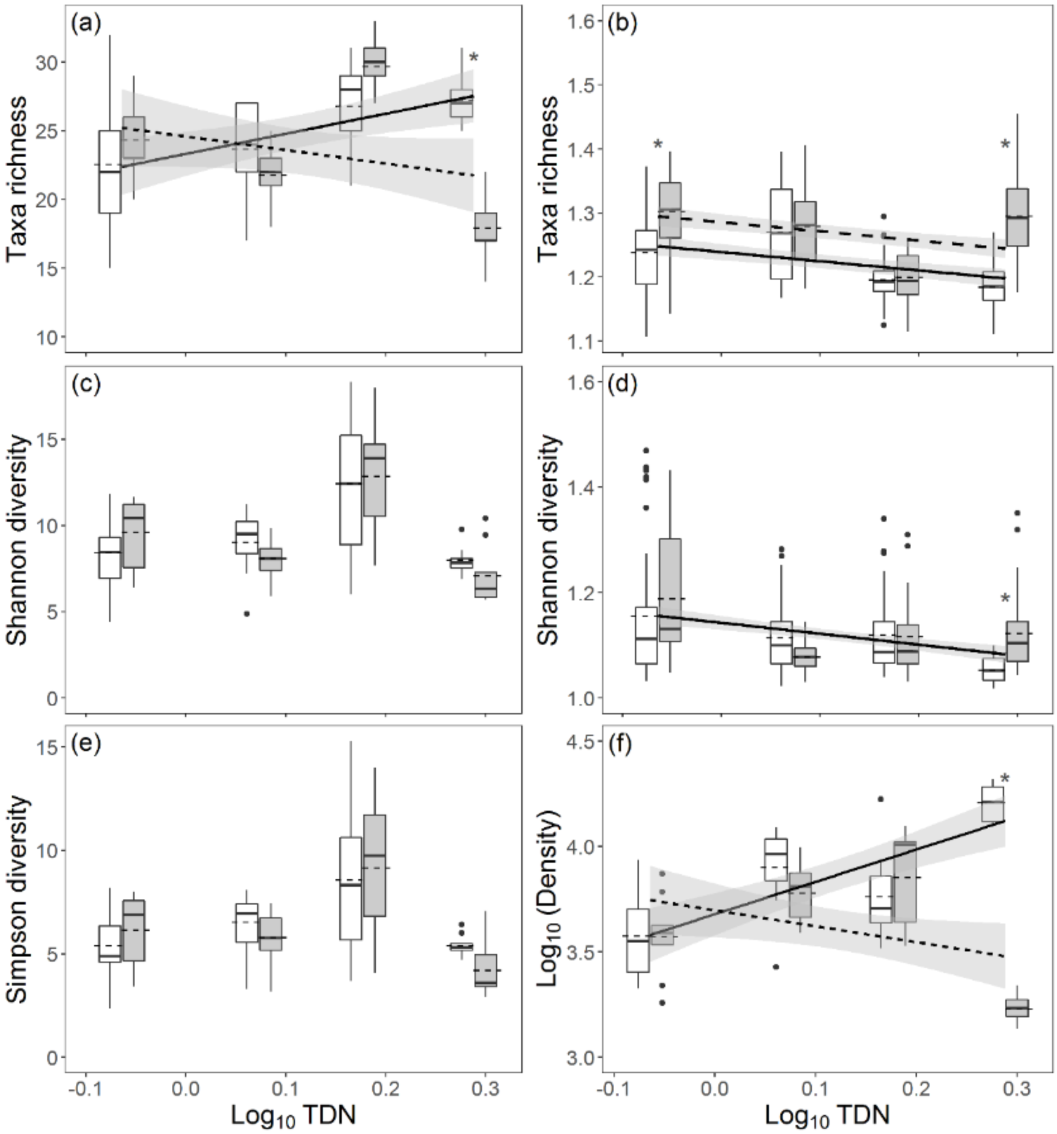


Fig. 3. Diversity and density of macroinvertebrate assemblage: alpha- and beta-diversity for taxa richness (a and b), alpha- and beta-diversity for Shannon diversity (c and d), alpha-diversity for Simpson diversity (e) and density (f). The box plots show the median, the interquartile range and the tails of the distribution. Dashed lines represent the mean value. White box plots refer to control reaches, grey box plots to impact. A single black regression line is represented when only the TDN gradient was significant and black regression lines (solid line for control; dashed line for diverted) are drawn when the effect of the diversion differed. Bands around the line represent the 95 % confidence interval. Significant differences between the control and diverted reaches within each river are marked with an asterisk.

Table 3 Linear model results comparing invertebrate density and diversity between reaches along the pollution gradient. Bold values indicate statistical significance ($p < 0.05$). Coefficients are shown for significant responses and consider pollution gradient (Log10TDN) and C reaches as reference in all cases.

		Log$_{10}$TDN			Reach			Log$_{10}$TDN:Reach		
		F	p	Coeff.	F	p	Coeff.	F	p	Coeff.
Taxa richness	Alpha	0.39	0.533		2.73	0.103		10.25	**0.002**	-24.46
	Beta	23.64	**<0.001**	-0.20	37.05	**<0.001**	0.05	3.22	0.074	
Shannon diversity	Alpha	0.01	0.939		0.01	0.937		0.63	0.429	
	Beta	29.6	**<0.001**	-0.26	2.53	0.113		2.04	0.154	
Simpson diversity	Alpha	0.0002	0.989		0.06	0.805		0.69	0.408	
Invertebrate density (log$_{10}$)		2.99	0.088		18.49	**<0.001**	-0.25	25.51	**<0.001**	-2.29

Table 4 Linear model results comparing energy fluxes throughout flux webs between reaches along the pollution gradient. Bold values indicate statistical significance ($p < 0.05$). Coefficients are shown for significant responses and consider pollution gradient (Log10TDN) and C reaches as reference in all cases.

	Log$_{10}$TDN		Reach			Log$_{10}$TDN:Reach		
	F	p	F	p	Coeff.	F	p	Coeff.
Total fluxes	0.14	0.714	6.02	**0.017**	-0.45	12.73	**<0.001**	-4.97
Herbivory	0.39	0.533	0.3	0.587		4.24	**0.043**	-3.43
Detritivory	0.11	0.739	6.86	**0.011**	-0.46	13.88	**<0.001**	-5.03
Carnivory	0.94	0.335	0.05	0.822		9.97	**0.002**	-5.93

Table 5 Linear model results comparing dependencies of trophic groups on basal resources between reaches along the pollution gradient. F and ps are identical within each trophic group as the sum of dependencies on biofilm and detritus equals 1. Bold values indicate statistical significance ($p < 0.05$). Coefficients are shown for significant responses and consider pollution gradient (Log10TDN) and C reaches as reference in all cases.

		Log$_{10}$TDN			Reach		Log$_{10}$TDN:Reach	
		F	p	Coeff.	F	p	F	p
Primary consumers	Biofilm	0.40	0.529		2.77	0.101	0.00	0.979
	Detritus	0.40	0.529		2.77	0.101	0.00	0.979
Omnivores	Biofilm	5.84	**0.018**	0.08	0.11	0.739	1.53	0.220
	Detritus	5.84	**0.018**	-0.08	0.11	0.739	1.53	0.220
Carnivores	Biofilm	0.98	0.326		0.12	0.727	0.19	0.663
	Detritus	0.98	0.326		0.12	0.727	0.19	0.663

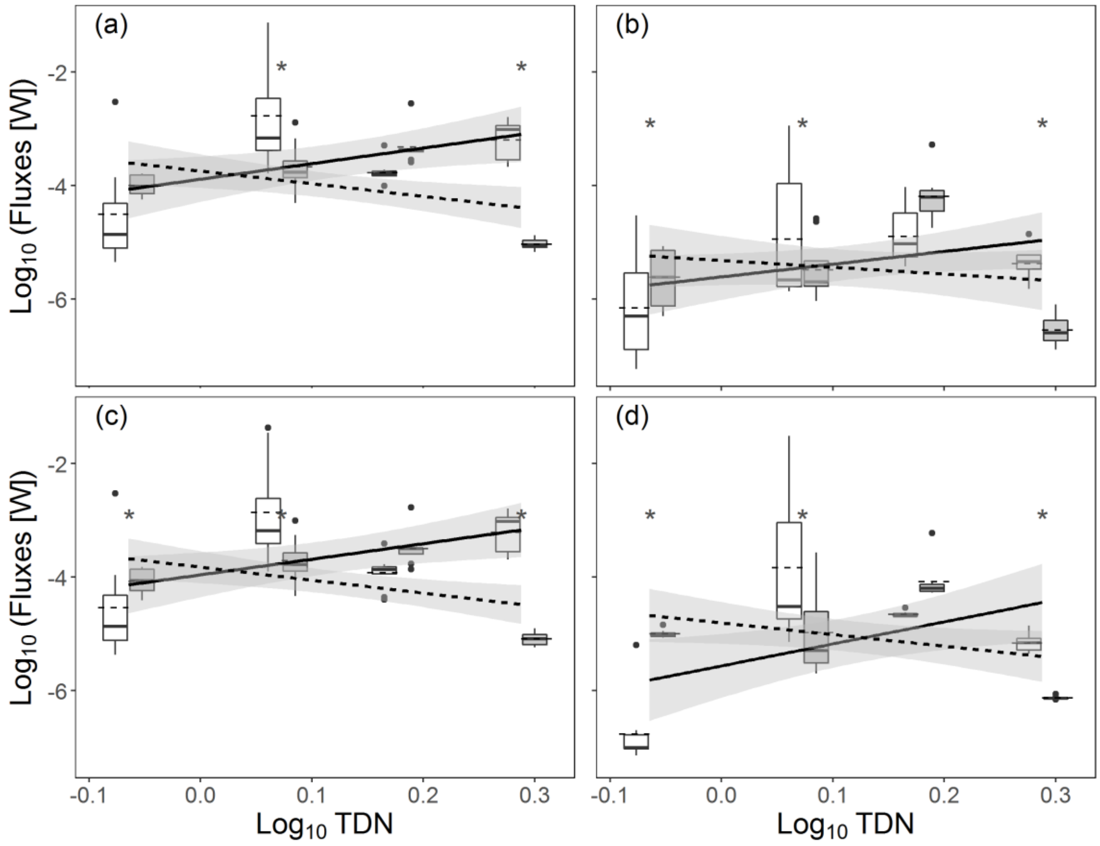


Fig. 4. Energy fluxes of stream food webs and functions: (a) fluxes throughout the entire food web and (b) herbivory, (c) detritivory and (d) carnivory related fluxes. The box plots show the median, the interquartile range and the tails of the distribution. Dashed lines represent the mean value. White box plots refer to control reaches, grey box plots to impact. A single black regression line is represented when only the TDN gradient was significant and black regression lines (solid line for control; dashed line for diverted) are drawn when the effect of the diversion differed. Bands around the line represent the 95 % confidence interval. Significant differences between the control and diverted reaches within each river are marked with an asterisk.

Alterations were exacerbated with both stressors

Interactive effects between both stressors were not observed on stock of basal food resources (Fig. 2, Table 2), although they were evident on some food web response variables. Along the gradient of pollution, alpha-diversity for taxa richness and invertebrate density increased in control reaches, whereas they showed a significant decreased in diverted reaches ($F_{1,68} = 10.25$, $p = 0.002$ and $F_{1,68} = 25.51$, $p < 0.001$; Fig. 3a and f, Table 3). Regarding fish assemblage, we did not observe a clear pattern in BM or in abundances as consequence of water diversion and pollution (Table S2). Mean BM and biomass of primary consumers, and MR of every trophic group (Table S1, Fig. S1) decreased in diverted reaches with increasing pollution. Interactive effects were also detected regarding energy fluxes along total food web and related to each function (Fig. 4, Table 4). In addition, herbivory and detritivory driven by primary consumers and omnivores and carnivory driven by carnivores also showed an interactive response of both stressors with decreasing energy fluxes in diverted sites along the pollution gradient (Fig. S2, Table S3). However, dependencies on basal food resources of each trophic group did not vary with both stressors (Table 5, Fig. 5). Size spectra of the entire community did not show differences either (Table 6, Fig. 6). Nonetheless, there were interactive responses of the intercept of primary consumers to pollution and diversion (Log_{10}TDN:Reach interaction, Table 6), but not of the slope (Body mass:Log_{10}TDN:Reach interaction, Table 6).

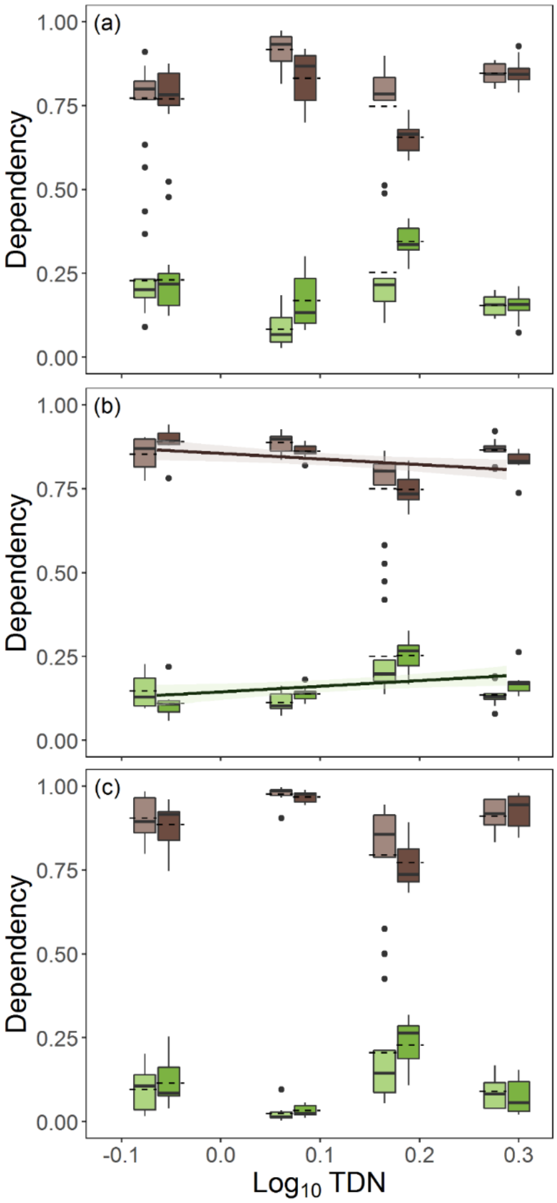


Fig. 5. Dependency of each trophic group ((a) primary consumers, (b) omnivores and (c) predators) on basal resources (detritus (brown boxes) and biofilm (green boxes)). The box plots show the median, the interquartile range and the tails of the distribution. Dashed lines represent the mean value. Light box plots refer to control reaches, dark box plots to impact. A single regression line is represented when only the TDN gradient was significant. Bands around the line represent the 95 % confidence interval.

Table 6 Linear model results comparing size spectra between reaches along the pollution gradient. Bold values indicate statistical significance ($p < 0.05$). Coefficients are shown for significant responses and consider pollution gradient (Log_{10}TDN) and C reaches as reference in all cases.

	Entire community			Primary consumers			Omnivores			Carnivores		
	F	p	Coeff.	F	p	Coeff.	F	p	Coeff.	F	p	Coeff.
Body mass	271.58	**<0.001**	-1.05	363.31	**<0.001**	-1.29	104.98	**<0.001**	-0.89	55.04	**<0.001**	-0.71
Log_{10}TDN	0.18	0.669		0.08	0.773		0.00	0.950		0.00	0.999	
Reach	0.26	0.611		16.71	**<0.001**	-0.33	2.13	0.151		0.69	0.411	
Body mass:Log_{10}TDN	0	0.970		0.55	0.461		1.25	0.270		0.01	0.926	
Body mass:Reach	0.43	0.517		0.08	0.785		0.20	0.658		0.08	0.779	
Log_{10}TDN:Reach	1.22	0.274		27.70	**<0.001**	-3.20	0.30	0.586		2.34	0.134	
Body mass:Log_{10}TDN:Reach	0.13	0.721		1.81	0.185		2.11	0.153		0.04	0.840	

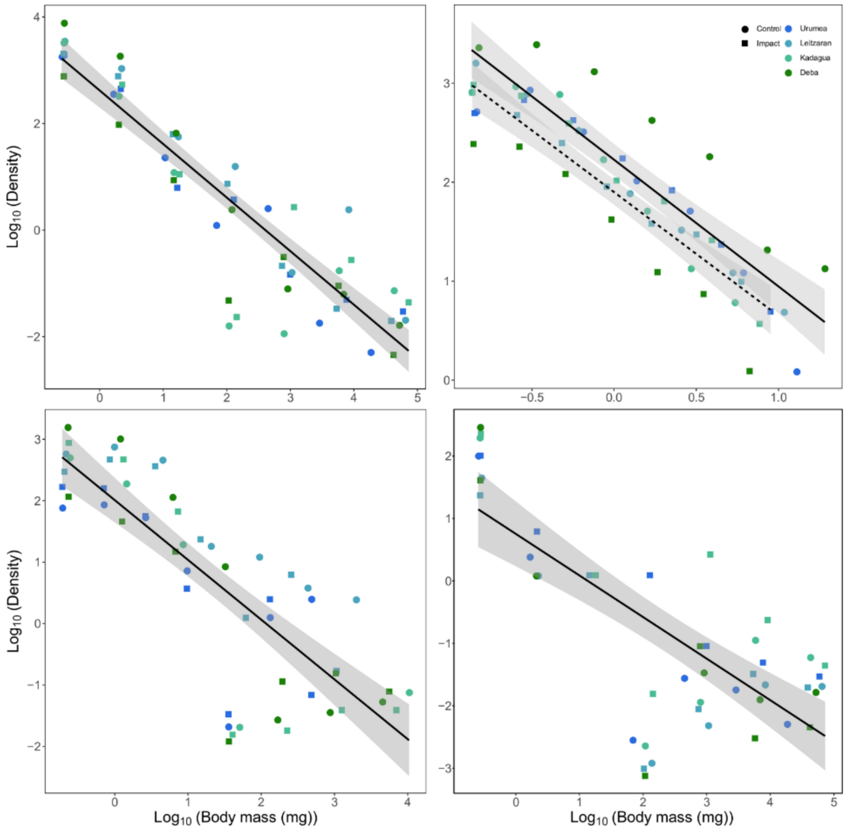


Fig. 6. Size spectra for (a) entire community, (b) primary consumers, (c) omnivores and (d) carnivores. Circles represent control reaches and squares diverted reaches. Regression lines are derived from the linear models. A single regression line is represented when only differences along body mass were significant and two regression lines (solid line for control; dashed line for diverted) are drawn when the effect of the diversion was significant. Note that the interaction pollution:reach was also significant in primary consumers, but only effect of diversion is shown.

Discussion

In the present study we have addressed the individual and joint effects water diversion and pollution, two pervasive stressors for freshwater ecosystems (Dudgeon *et al*., 2006), produced in the structure and energy transfer of stream food webs. Water diversion reduced the abundance of detritus, whereas nutrient pollution promoted biofilm production. These changes at the base of the brown and green food webs were propagated to higher trophic levels, but their effects differed with each stressor. Moreover, water diversion increased community heterogeneity, and reduced total energy fluxes and detritivory. Water pollution, on the other hand, decreased heterogeneity of invertebrate communities and increased herbivory, carnivory and dependency on biofilm within omnivores, however it did not change energy transfer efficiency. Interactive effects of both stressors were significant among most of the response variables.

The effects of water diversion at the base of the food web induced changes in energy fluxes

Several studies have observed a decrease in detritus stock in diverted reaches (Casas *et al*., 2000; Martínez *et al*., 2013), a consequence of its retention in the impoundments above weirs (Schmutz and Moog, 2018) and of its diversion towards canals (Arroita *et al*., 2015). Biofilm, on the other hand, can be affected by water velocity and river flow which can be altered by water diversion. Increased water velocity enhances nutrient exchange, and thus, biofilm growth (Dewson *et al*., 2007), but can also limit biofilm accrual due to excessive shear forces (Hondzo and Wang, 2002). In our study however, water diversion did not significantly affect biofilm biomass. Despite the few instant velocity measures we had were not enough to properly assess differences among reaches, they suggested a lack of differences from control to diverted sites, which is in line with the lack of differences in biofilm biomass.

Water diversion did not affect diversity at alpha scale, however, it increased taxa richness at beta-diversity scale (i.e. heterogeneity among samples) from control to diverted reaches. On the contrary, invertebrate density decreased downstream from dams. Many studies have observed alterations on invertebrate communities downstream from these structures (González and Elosegi, 2021; Kuriqi *et al*., 2021; Linares *et al*., 2019; Munasinghe *et al*., 2021). González and Elosegi (2021) for instance, observed a decrease in total invertebrate abundance per channel unit length with water abstraction although they did not observe a decrease in total density, whereas Munasinghe *et al*. (2021) reported a decrease in richness downstream from weirs. The available habitat reduction induced by flow regulation has been identified as one of the main drivers for reduced invertebrate abundances (Brasher, 2003; Martínez *et al*., 2020). Decreased habitat availability is not the only consequence of flow regulation affecting communities, it also reduces habitat diversity (Cazaubon and Giudicelli, 1999). Moreover, dams disrupt the continuity of sediment transport reducing the sediment input in the downstream reaches (Tena *et al*., 2011); hence, we expected a more homogeneous invertebrate community downstream from dams. In the present study, however, we did not gather enough evidences to demonstrate an increase in homogeneity of diverted reaches, and beta-diversity for taxa richness increased downstream from dams. This result could be explained by the decrease in invertebrate density, as beta-diversity generally declines with increasing number of individuals (Stier *et al*., 2016). Richness differences, one of the processes shaping beta diversity patterns (Carvalho *et al*., 2013), could have also increased beta-diversity downstream from dams. The loss in density could have led to an increase in the probability of competitive exclusion, with extinctions of different rare species occurring in each local community. However, the increased beta-diversity downstream from dams could additionally suggest that the impact of water diversion was not strong enough to decrease beta-diversity, since decreases in beta-diversity can be consequences of strong deterministic effects of disturbances (Chase, 2007). Furthermore, reductions in fish abundance and richness are frequently reported as consequence of the interruption of longitudinal connectivity, alteration of the flow regime between control and diverted reaches and change of biophysical habitat conditions caused by small weirs (Kuriqi *et al*., 2021). However, the presence of the same fish species in upstream and downstream reaches of our study with higher fish densities in most of the diverted sites also suggest that water diversion in our study did not cause a strong impact on fish assemblages.

Nevertheless, we observed a decrease in detritivory downstream from dams, especially within primary consumers, as a response to the decrease of detritus stock. Decreases in organic matter breakdown downstream from diversion dams have been observed (Arroita *et al*., 2017), which suggests that the reduced stock of organic matter in river reaches facing water diversion could lead to a reduction of energy transfer into higher trophic levels, affecting stream productivity and modifying the trophic structure. In these streams, as primary production is usually limited by nutrients (Elser *et al*., 2007; Tank and Dodds, 2003) and canopy cover (Bernhardt *et al*., 2018), primary consumers use organic matter as the main energy source (Vannote *et al*., 1980; Zhang *et al*., 2019). In the current work we have demonstrated that the reduction of the detritus stock from control to diverted sites was linked to the decrease in energy transfer through the brown food web as well as in entire food web. The reduction of detritus was also related to a lower size independent abundance of primary consumers, i.e. a lower intercept of the size spectra for primary consumers. These results go in line with the observations of Larrañaga *et al*. (*In prep.*) who reported a lower intercept in shredders' size-spectra with lower resource abundance. Nonetheless, the change we observed in the energy transfer was not followed by a modification of dependencies for basal resources. Additionally, we observed no variation in community size spectra with water diversion, and the energetic equivalence rule (i.e. energy being equally transferred among different body size categories) was followed in all cases (Damuth, 1981).

Moderate pollution increased stock of basal food resources, but did not alter energy transfer

In literature a non-linear biofilm production is frequently described with nutrient pollution, reporting stimulatory effects at moderate levels of nutrient loads (e.g. Ardón *et al*., 2021; Dodds and Smith, 2016; Ribot *et al*., 2015). Stimulatory effects of other pollution sources on biofilm have also been reported (Pereda *et al*., 2021, 2020, 2019), which suggests that subsidy effects of nutrients can override the toxic effects of other compounds, at least for the most resistant biofilm taxa (Rosi *et al*., 2018). Similarly, along our moderate pollution gradient, biofilm production was promoted. However, contrary to what we expected, we cannot describe an increase in the relative abundance of this higher quality resource over detritus, since the biofilm:detritus ratio was maintained along the gradient. Still, although we did not assess the quality (i.e. C:N ratios) of basal resources in the current study, an increase could be expected as many studies have described an increase in detritus and biofilm quality with water nutrient enrichment (Evans-White and Halvorson, 2017; Scott *et al*., 2008; von Schiller *et al*., 2007; Xu and Hirata, 2005) .

For macroinvertebrates, on the other hand, a decrease in diversity and taxa richness has been frequently reported with increasing pollution with urban origin (Arimoro and Keke, 2017; Cortelezzi *et al*., 2019; Johnson *et al*., 2013; Tagliaferro *et al*., 2020). Even at low water-quality degradation levels some sensitive species can disappear (Cortelezzi *et al*., 2013). In our experiment, however, we did not observe the expected decrease in alpha-diversity with increasing pollution. Notwithstanding, we observed a homogenization of invertebrate assemblages since beta-diversity for taxa richness and for Shannon diversity decreased along the pollution gradient. Increasing nutrient concentrations have already been related to homogenization of invertebrate assemblages (Johnson and Angeler, 2014), but also to increased productivity (Ardón *et al.*, 2021). Thus, we anticipated an increase in invertebrate abundance with the promoted biofilm biomass in our moderate-pollution gradient. However, although we did not observe a significant increase in densities with nutrients as in other studies (Cross *et al.*, 2006; García *et al.*, 2017; Niyogi *et al.*, 2007), we observed an increase in primary consumers' and omnivores' mean BM, together with an increase in omnivores' total biomass. Increased shredders body size with nutrient enrichment has already been attributed to enhanced detritus quality via microbial assimilation (García *et al.*, 2017), which also stimulates biomass production of primary and secondary consumers (Cross *et al.*, 2006; Davis *et al.*, 2010; Demi *et al.*, 2018). However, we cannot discard combined effects of resource quality and quantity to be the responsible of these results, as we also observed higher resource abundance along the pollution gradient.

We expected an increase in energy fluxes along the pollution gradient as consequence of an increment in detritus quality by increased nutrient concentration (Cross *et al.*, 2003), but especially by the increased biofilm biomass, a higher nutritional quality resource than detritus (Cross *et al.*, 2005) which can sustain a high production of primary consumers (McCutchan and Lewis, 2002) as they might prefer it against the allochthonous resource (Bumpers *et al.*, 2017). A shift from brown to green pathway through the increase of the abundance of herbivores with nutrient enrichment has been observed for system where detritus was the main basal resource (Bumpers *et al.*, 2017). However in our study, despite the increase in herbivory driven by omnivores whose dependency on biofilm also increased, we did not observe neither a general increase in the energy fluxes nor a promotion through the green food web, although an increase was observed in the control reach which was concealed by the opposite trend in the impact reach.

Thus, as the increase in basal resource quality (i.e. a decrease in C:nutrient ratio) with nutrient enrichment (Evans-White and Halvorson, 2017) reduces the stoichiometric gap between resources and consumers, and increases the efficiency of trophic transfer (Mulder and Elser, 2009), we predicted shallower slopes in the size spectra with food webs holding relatively more large-individuals along the pollution gradient. As mentioned before, we did not assess the C:N ratios of detritus and biofilm in the present study. Nevertheless, other studies that report size specific responses to pollution show that small bodied organisms can be more sensitive to toxicity (Gergs *et al.*, 2015; Kang *et al.*, 2019; Taddei *et al.*, 2021) due to a higher specific metabolism and a greater surface to volume ratio (Wang and Zauke, 2004). This toxic effect on smallest body size categories should turn slopes of size spectra shallower. However, we did not observe any alterations in the size spectra. This can be either an outcome of both the toxic and the subsidy effect to be negligible, or of both effects cancelling each other. The observed responses on other variables to water pollution suggests that effects were not negligible and both toxicity and subsidy played a role in keeping the size spectra unchanged.

Pollution shaped the response of food webs to water diversion

In studies addressing the effects of multiple stressors, it is convenient to classify the stressors depending on the ecological relevance they have and to describe the interactive effects they induce on the response variables. Although to our knowledge the amount of studies addressing the combined effects of water diversion driven by low weirs and pollution on food webs are scarce (e.g. de Guzman *et al*., 2021), there are few more studies addressing the effects of flow reduction and nutrient enrichment (Lange *et al*., 2014; Matthaei *et al*., 2010), which are consequences of water diversion and pollution. Matthaei *et al*. (2010) for instance, observed stronger pervasive effects of flow reduction on benthic invertebrates and algal biomass than those related to nutrient enrichment. Lange *et al*. (2014) on the contrary, described larger effects of nutrient pollution than water diversion on fish populations in a field study. Such different outcomes are the results of the varying impact both stressors of continuous nature can produce depending on the intensity of each single stressor. Local extinctions for instance, might be driven by intense nutrient pollution and even droughts caused by severe water diversions can have devastating effects on communities. Contrary to what de Guzman *et al*. (2021) observed, in the present study the variables of interest seemed to be more sensitive to water diversion, a response which was often modulated by water pollution. Regarding macroinvertebrate community for instance, a decrease in density in the diverted sites was observed, which was intensified with increasing pollution. Moreover, taxa richness at alpha-diversity scale, a variable unaffected by each stressor independently, was also strongly and negatively affected in combination of water diversion and pollution. Energy fluxes of the entire community and fluxes related to each function also followed the same tendency, showing a decrease in diverted sites along the pollution gradient. Finally, size spectra only showed a size independent decrease with diversion, which was intensified with pollution. Thus, in the current study, pollution exacerbated the effects of water diversion, and although we expected an increased importance of the relatively more abundant biofilm, water diversion and the decrease in organic matter was the main resource shaping the structure of communities and energy fluxes along food webs.

Conclusions

Studying the co-occurring multiple stressors to properly understand their interactive effects and classify their relevance according to their impact in ecosystems is crucial to accurately manage freshwater ecosystems. In the current study addressing the effects of water diversion and pollution on stream food webs, we observed that each stressor independently affected different features of food webs. However, when analysing their impact simultaneously, pollution intensified the effects of diversion in the variables of interest. We anticipate similar findings in freshwater ecosystems that mostly depend on detritus. However, the intensity of each stressor can vary. Thus, different outcomes might also be expected with larger water removals or more severe levels of pollution which can trigger local extinctions altering food web composition and energy fluxes in different manners. Therefore, as it is uncertain to what extent the non-linear responses to both stressors will emerge with their increasing intensity (Hillebrand *et al*., 2020), we consider the current study will promote further research on the interactive effects of multiple stressors of varying intensities to better understand their effects on freshwater ecosystems globally.

Acknowledgements

This research was supported by the Spanish Department of Economy, Industry and Competitiveness through the project GL2016-77487- R (DIVERSION), the European Social Fund, the Basque Government (Consolidated Research Group IT951-16) and the Biscay Province Council (61/2015). Authors also acknowledge the financial support from pre-doctoral fellowship from the Basque Government (I. de Guzman), pre-doctoral fellowship from the Spanish ministry (A.V. Perez-Calpe). D. von Schiller is a Serra Húnter Fellow. J. M. Montoya is funded by the FRAGCLIM Consolidator Grant (number 726176) from the European Research Council under the European Union’s Horizon 2020 Research and Innovation Program and by the ‘Laboratoires d’Excellences (LABEX)’ TULIP (ANR-10-LABX-41). Authors are especially grateful to Janire Diez, Maria Blanco, Lukas Iruretagoyena and Miren Atristain for their assistance in field and laboratory analyses, to Benoit Gauzens and Ulrich Brose for their advice on calculations regarding energy fluxes, to EKOLUR (Asesoría ambiental-Ingurumen aholkularitza), SGIker technical and human support (UPV/EHU, MICINN, GV/EJ, ESF).

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Chapter 3

Treated and highly diluted wastewater promotes the green food web but reduces trophic diversity

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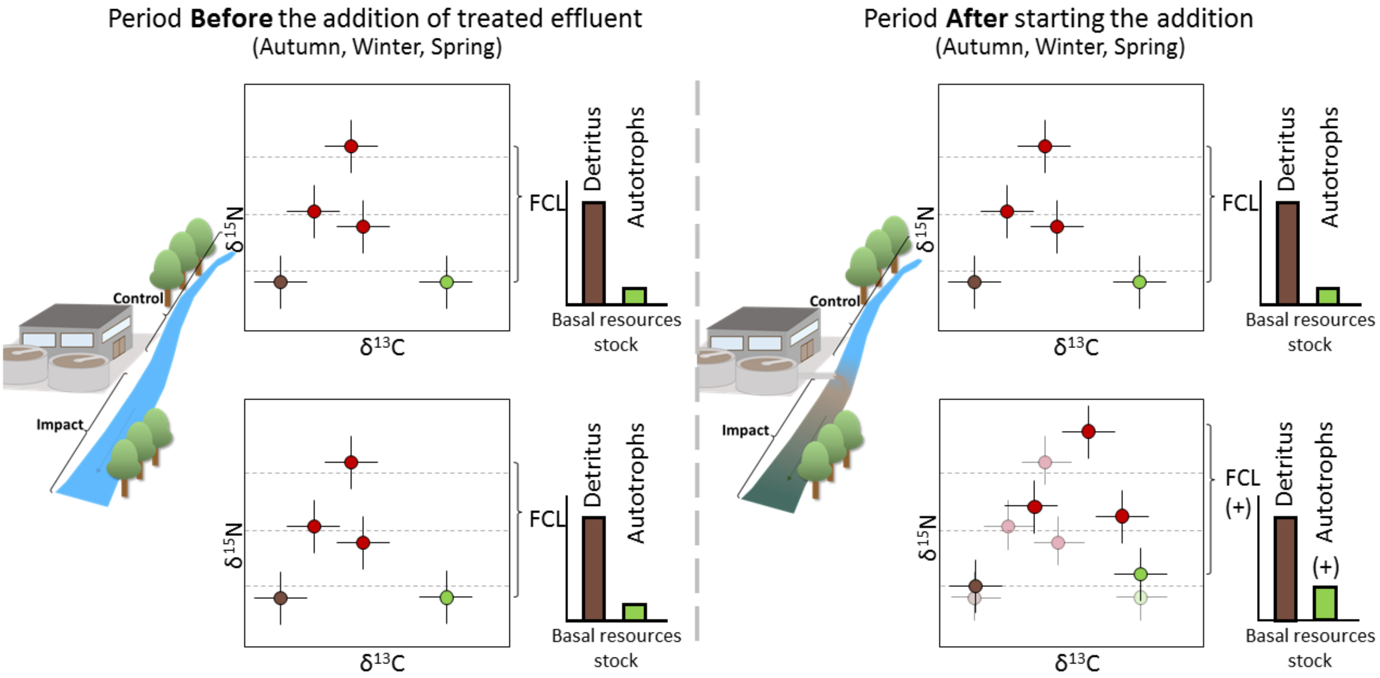
Abstract

Human activities have increased the amount of pollutants entering world rivers. In many regions, the implementation of Wastewater Treatment Plants (WWTPs) has greatly improved the quality of point source inputs, enhancing river ecological status. Nowadays, most rivers in developed regions receive WWTP effluents, which still contain a complex cocktail of pollutants whose joint effects are poorly known. We conducted a whole ecosystem manipulation experiment following a BACI design (Before-After/Control-Impact), where we diverted part of the treated effluent of a large WWTP into a small, unpolluted stream to assess its effects on food webs. Although previous studies described the low toxicity of the effluent, we still expected changes in the complexity of food webs. We sampled basal food resources, invertebrates and fish and we hypothesized an increase in autochthonous resource contribution into the diets of primary consumers due to the relative increase in biofilm availability over detritus, as well as an increase in food web complexity, by increasing food chain length and trophic diversity. Although coarse detritus was generally the main contributor to the diet of primary consumers, with the addition of the effluent, we observed a promotion in the green pathway paralleled to an increase in biofilm biomass during autumn and winter. However, contrary to what we expected the addition of the effluent reduced trophic diversity in these seasons and maximum food chain length was not altered. The present study reveals that small impacts can modify ecosystem productivity, affecting green and brown food web pathways, which might end up altering food web composition and associated ecosystem functioning. Thus, this experiment suggests that current methods for treating polluted waters might be insufficient to preserve natural properties of food webs.

Key words: *BACI experiment, ecosystem-level manipulation, freshwater food webs; pollution, treated wastewaters, food web complexity, stable isotopes*



Introduction

The growth of global population and the socio-economic development in the last decades has drastically induced an increase of urbanization, with over 55% of the world population living in urban areas (United Nations, 2019). In order to deal with anthropogenic pollution entering freshwater ecosystems, Waste Water Treatment Plants (WWTP) have been widely implemented. Although these measures have greatly decreased the amount of contaminants reaching aquatic ecosystems (Vaughan and Ormerod, 2012; Brion *et al*., 2015), treated sewage still consists of a complex mixture of nutrients, organic matter, pollutants and pathogens (Pascual-Benito *et al*., 2020). Some of these compounds are invariably toxic (Patel *et al*., 2020; Vasilachi *et al*., 2021), whereas others can subsidize biological activity, although they can also become hazardous at high concentration (Carey and Migliaccio, 2009; Wang *et al*., 2019), for instance, nutrients. Therefore, effluent effects on freshwater ecosystems strongly depend on their composition and on dilution rate, with larger detrimental effects when dilution is low (Carey and Migliaccio, 2009; Arenas-Sánchez *et al*., 2016).

In small forested streams detritus, mainly as leaf litter, is the main food resource (Vannote *et al*., 1980; Zhang *et al*., 2019) since primary production is usually limited by canopy cover (Bernhardt *et al*., 2018) and nutrients (Tank and Dodds, 2003; Elser *et al*., 2007). This detrital organic matter colonized by microbes, constitutes the base of what is coined brown food webs (Marks, 2019). Nonetheless, algae, a more scarce but higher quality basal food resource, play also a key role in these ecosystems (Brett *et al*., 2017) having important consequences for both green and brown pathways of food webs (Marcarelli *et al*., 2011; Crenier *et al*., 2017) as they that can stimulate or inhibit detritus consumption (Halvorson *et al*., 2016, 2019). With the release of resources such as nutrients or organic matter into freshwaters, WWTP effluents can stimulate the productivity of ecosystems and induce bottom-up cascades altering composition and structure of food webs. The increase in nutrient concentration such as nitrogen and phosphorous can reduce the constrain for algal growth (Marcarelli *et al*., 2009) and increase their biomass (Keck and Lepori, 2012). These changes at the base of the food web enhance initial energy flow through the green pathway (Canning and Death, 2021) which can be propagated towards higher trophic levels (Ardón *et al*., 2021) altering food web structure and increasing trophic diversity (García *et al*., 2017). Furthermore, species population size at high trophic levels is limited by the energy loss within each trophic transfer (Hutchinson, 1959; Pimm, 1982). With the moderate increase of nutrients, toxic effects can be contained, but the stoichiometric gap between food resources and their consumers is narrowed increasing trophic transfer efficiencies (Mulder and Elser, 2009) and thus easing energy transfer toward higher trophic levels (Pimm, 1982; Schoener, 1989). Therefore, more productive ecosystems that do not have to cope with the toxic consequences of eutrophication, should allow longer food chains.

The strong effects of poorly treated and highly concentrated WWTP effluents on ecosystems are evident and have been widely studied (e.g. Morrissey *et al*., 2013; Mor *et al*., 2019; Hamdhani *et al*., 2020). However, the effects that well-treated and highly diluted effluents may cause have not been so well investigated since freshwaters are usually exposed to co-occurring stressors or because it is complicated to isolate their effects from confounding factors related to spatiotemporal variability. Still, as even low pressures can produce clear changes on ecosystems (Hillebrand *et al*., 2020), these weaker but widespread stressors could induce significant changes in food web properties. Therefore, sound experimental designs that adequately isolate the effects of interest are necessary to analyse and discern the impacts of these less evident pressures. In this work, we carried out a whole ecosystem manipulation experiment which followed a BACIP (Before-After/Control-Impact/Paired) design (Downes *et al*., 2002), by diverting part of the treated effluent of a WWTP into an unpolluted adjacent stream to assess its effects on food web organization and trophic niche distribution. The effluent used showed low toxicity for microbial performance and detritivore growth in a previous study (Solagaistua *et al.*, 2018). Therefore, we did not anticipate large detrimental effects on food web complexity. We hypothesized an increase in autochthonous resource contribution to consumers with the addition of the effluent as consequence of the relative increase in biofilm availability over detritus. In addition, we expected the effluent to increase food web complexity, by increasing food chain length and trophic diversity, with lowered redundancy (Fig. 1).

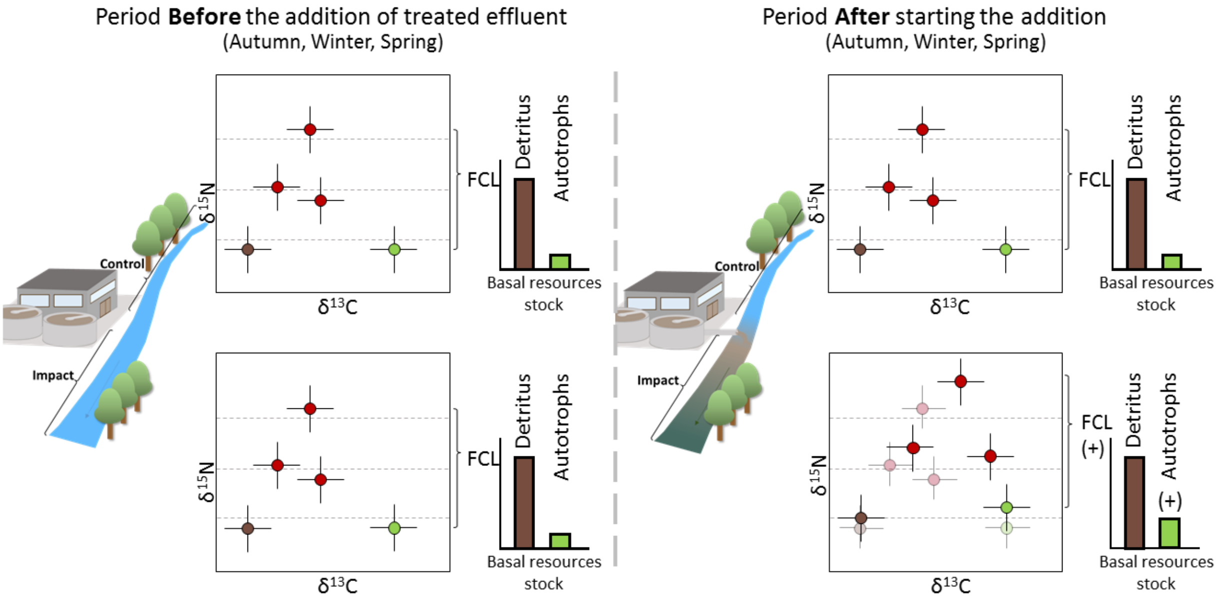


Fig. 1. Conceptual figure of the BACI (Before-After-Control-Impact) experimental design and the proposed hypotheses. The Control reach is upstream from the effluent pouring location. The Impact site receives treated effluent during the After period. FCL refers to food chain length. Basal food resources, detritus and autothrops, are represented in dark brown and light green respectively in the figure, and consumers are represented in dark red. The food web arrangement of the C-N biplots from the sites not receiving the effluent is redrawn in ligther colours in the effluent-receiving biplot as reference. $\delta^{15}N$ indicates the trophic position of each element of the biplot, whereas $\delta^{13}C$ informs about the relative proximity to the basal resources. (+) symbol indicates an increase.

Materials and methods

Study site and experimental design

The experiment was carried out in the Apraitz stream (N Iberian Peninsula, 43°13′41.1″N 2°23′56.3″W), a small unpolluted river with a mean discharge of 0.12 m^3/s draining a 7 km^2 catchment over sandstone and shale. In the lowermost 300 m, its riverbed is dominated by bedrock and cobbles and the young riparian forest surrounding it, is mainly composed of black alder (*Alnus glutinosa* (L.) Gaertn.), hazel (*Corylus avellana* L.) and ash (*Fraxinus excelsior* L). This reach runs nearby the WWTP of Apraitz, which releases the treated sewage into the Deba River (mean discharge during our study 10.9 ± 0.7 m^3/s, http://gipuzkoa.eus/) through a regularly pulsed discharge (20-40 minutes every 2h) 10 metres upstream the site where the Apraitz stream flows into the Deba River. The WWTP receives the sewage of approximately 90.000 population equivalents from the adjacent urban and industrial areas, treating on average 29.904 m^3 of wastewater per day in its sequential biological reactors (https://www.acciona-agua.com). After mixing the sewage with activated sludge and subjecting it to aerobic and anaerobic conditions to reduce the load of organic matter and nitrogen, it receives tertiary treatment (precipitation with ferric sulphate) to reduce phosphorous concentrations.

The experiment followed a BACIP (Before-After Control-Impact Paired) design, which allows controlling temporal and spatial variability to isolate the effect of the impact of interest with samples paired in time (Downes *et al*., 2002). During a year we diverted part of the WWTP effluent into the lowermost 150 m of the Apraitz stream to get a final dilution rate similar to that of the effluent discharged into the Deba River (0.2-4 % and 0.1-9 % of effluent concentration, respectively (Pereda *et al*., 2020)). We defined two 100-m long reaches: a Control reach, upstream from the effluent addition site and an Impact reach just below the discharge (Fig. 1). We sampled at both reaches in autumn, winter and spring before and after the start of the effluent addition (May 2017). The effluent release changed significantly water physico-chemical characteristics at the impact reach (Pereda *et al*., 2020): dissolved oxygen saturation and pH decreased by 10% but temperature remained unaffected. Ammonium and soluble reactive phosphorous increased 5.2 and 2.4 times respectively.

Effluent characteristics in the current study

Data regarding physicochemical characteristics of treated effluent shown in this study was collected by managers of the WWTP of Apraitz. Effluent characteristics such as pH, conductivity, Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and Total Nitrogen (TN), differed among seasons, whereas Total Phosphorous did not vary. Conductivity, COD and TN showed the highest values during Autumn, and pH and BOD in Spring (Table S1). Measurements of $\delta^{15}N_{NO3}$ in effluent and stream water samples collected during the present study (n=5 and n= 2 respectively; 250 mL each), showed enriched values of $\delta^{15}N_{NO3}$ in effluent (20.41 ± 4.38) over stream water (6.44 ± 0.37) (methodology described in Bujak *et al*. (2021)). In addition, we calculated effluent contribution by placing a water level datalogger (Solinst Levelogger Edge 3001; Solinst Canada Ltd., Georgetown, USA) per reach and correcting level data for the atmospheric pressure (Barologger, Solinst Levelogger Edge 3001) to regress it against discharge calculated from the time-EC curves to obtain continuous discharge and to compare it between both reaches. During the sampling occasions of the present study, effluent contributed on average in 3.64% just before the Autumn sampling (Table S1), showing a significantly higher contribution than in periods just before Winter and Spring samplings.

Sampling and sample processing

Stock of basal food resources

Prior to the start of the experiment we deposited 50 artificial substrata (granite paving stones of 20 x 10 x 8 cm) along each reach (covering less than 0.5% of the streambed) to allow for biofilm colonization. The colonising biofilm was formed by a similar amount of photoautotrophic and heterotrophic microorganisms, with a tendency to net autotrophy according to Pereda *et al*. (2020). In each sampling campaign and reach, we scrapped the whole surface of five randomly chosen paving stones, and processed them to obtain ash-free dry mass (AFDM) per surface unit (g m^{-2}) (complete methodology in Pereda *et al*. (2020)). In addition, we collected nine coarse detritus samples per reach with a Surber sampler (0.09 m^2), and we processed the organic matter retained on an 8-mm sieve. We separated leaves from the rest of coarse detritus and processed each category to obtain AFDM in each sample (see organic matter processing in Pereda *et al*. (2020)). Consumption of wood and other recalcitrant materials by macroinvertebrates is negligible compared to leaves (Díez *et al*., 2002; Gulis *et al*., 2008), thus we only consider leaves (hereafter coarse detritus).

Stable Isotope Analysis

We sampled the entire food web in each reach and occasion for stable isotope analysis (SIA). We collected all the available basal food resources from each reach: biofilm, fine detritus, coarse detritus (leaves of every terrestrial origin species), bryophytes and filamentous green algae. Six composite samples of biofilm were collected in each reach by scrapping the whole surface of nine cobbles and collecting the slurry in filtered river water (0.7 µm pore size, Whatman GF/F). Six samples of fine detritus were randomly collected per reach in each sampling campaign using a sediment corer (surface 81.7 cm^2). The remaining basal resources were individually gathered from the riverbed. Macroinvertebrates were collected with a kick sampler (0.5 mm mesh aperture) in six transects along each reach, sorted, rinsed and identified in the field to genus-level (except for some Diptera identified to subfamily level and Annelida to subclass level) following (Tachet *et al*., 2010). We collected up to nine invertebrate samples per taxon in each reach, containing from one to 55 individuals depending on their body mass. Mollusks were extracted from their shells, and when possible, the digestive tracts of the predators were removed, as gut contents can affect the isotopic signature of the sample (Mateo *et al*., 2008). Finally, we carried out fish sampling along the 100-m long reaches by depletion electrofishing with a backpack-electrofishing unit with variable output current (MARTIN PESCADOR III, Alborlan S.L.). Stop-nets were set at the upstream and downstream ends of the reaches. All cached fish were anaesthetised with MS-222, identified and up to five individuals per species in each reach were euthanized (reference number of the ethics commission: M20/2016/177). Samples of dorsal muscle were extracted in the field. All the samples were immediately frozen (-20°C) for processing and SIA.

We freeze-dried frozen samples (VirTis Benchtop 2K) (from 12 to 72h depending on their water content), grounded (Resources in a ball-mill (Vibration mill MM301, Fisher Bioblock Scientific); Animal samples in a homogeniser (Precellys® 24, Bertin instruments)) and weighed them (approximately 1 mg for invertebrates and fish, 10 mg for fine detritus and 2 mg for other basal resources) into tin capsules (Lüdiwiss Sn 98, 5 x 8 mm) for SIA. Elemental concentrations of δ^{13}C and δ^{15}N were analyzed at the Helmholtz-Zentrum für Umweltforschung (Magdeburg, Germany) by a flash combustion on a Flash 2000 elemental analyzer connected to a Delta V isotope ratio mass spectrometer operated in the continuous helium flow mode via ConFlo IV split interface (EA-IRMS; Thermo Fisher Scientific, Bremen, Germany). Three in-house standards were analyzed every 15-16 samples for quality assurance. Results are expressed as relative difference between ratios of samples and international standards (Pee-Dee Belemnite for δ^{13}C, atmospheric N for δ^{15}N) and expressed in per mille delta notation [e.g. $\delta 13C$ (‰) = $(Rsample/Rstandard - 1)1000$] (Fry, 2006). Analytical error (mean SD from in-house standards) associated with our sample runs was estimated at 0.2 ‰ for δ^{13}C and 0.3 ‰ for δ^{15}N.

Data treatment

Trophic position and maximum food chain length

Maximum food chain length (the linear trophic distance between basal resources and consumers) in each site was estimated following the maximum trophic position convention, assessing trophic positions (TP) of fish and comparing their δ^{15}N values to the mean δ^{15}N value of the basal resources at each sampling site (Cabana and Rasmussen, 1996): $TP = \frac{(\delta^{15}N_{top\,predator} - \delta^{15}N_{baseline})}{3.4} + \lambda$. Where *3.4* is the trophic discrimination factor (TDF) of δ^{15}N (Vander Zanden and Rasmussen, 2001; Post, 2002) and λ the trophic level of the baseline indicator, set as 1 because primary producers were used as the baseline. We obtained the maximum food chain length from the mean trophic position values of the individuals with the highest trophic position in each reach (Table S2). We also searched for differences in the trophic position of *Phoxinus bigerri* (Kottelat, 2007) and *Salmo trutta* (Linnaeus, 1758).

Contribution of resources to the diet of primary consumers

We used Bayesian Mixing Models to estimate the contribution of basal resources to the diets of the primary consumer invertebrates at each sampling occasion and reach using the MixSIAR package (Bayesian Mixing Models in R; (Stock and Semmens, 2013). Autochthonous resources, fine detritus and coarse detritus were treated as separate resources. We merged together biofilm, filamentous green algae and bryophytes into the autochthonous resources category due to the low and sparse distribution among sampling reaches of filamentous green algae and bryophytes. The models consider uncertainty and variation in consumers and TDF to generate a distribution of possible mixing solutions based on the available resources. MixSIAR also provides error terms that contemplate variation due to sampling processing and due to consumers' variability itself (i.e. individual differences in digestibility, assimilation efficiency and metabolic rates) (Stock and Semmens, 2016). We used TDF and uncertainties specific for aquatic invertebrates (0.1 ± 2.2‰ for δ^{13}C and 2.6 ± 2.0‰ for δ^{15}N (Brauns *et al.*, 2018)). Concentration dependence (Phillips and Koch, 2002) and a multiplicative error structure (Stock and Semmens, 2016) were also considered in the models. Posterior estimates of the proportional contribution of each resource to primary consumer's diet were obtained for each reach. Consumer stable isotope data was previously checked for outliers through simulated mixing polygons (Smith *et al.*, 2013) with the packages sp (Pebesma and Bivand, 2005) and splancs (Bivand *et al.*, 2017). The method uses a Monte Carlo simulation to iterate Convex hulls ('mixing polygons') based on means and SD of source data and

TDF. It applies the point-in-polygon assumption to test if source contributions can explain consumer's isotopic signature in the proposed mixing model. We excluded samples not following the recommendations by Smith *et al*. (2013), 0.23% of the primary consumer samples had to be excluded.

Community iso-space metrics

The trophic structure of the consumer community was estimated for each sampling occasion and reach using the community-wide metrics described in Layman *et al*. (2007a) and Jackson *et al*. (2011). We considered four functional groups: primary consumers, omnivores, predatory invertebrates and fish. Some metrics consider the distribution of the components of each community in the $\delta^{13}C$ - $\delta^{15}N$ space to inform about the trophic diversity within each food web. Mean distance to centroid (CD) is one of these metrics, which provides information on the trophic niche through the species distribution in the iso-space. Trophic redundancy was estimated using mean nearest neighbour distance (MNND) and standard deviation of the nearest neighbour distance (SDNND). MNND is the main metric representing trophic redundancy, which provides a measure of density and grouping of the community members. SDNND informs about the evenness of spatial density and packing. Smaller MNND represents food webs with taxa having more similar trophic ecologies, whereas smaller SDNND indicates a more uniform spacing of taxa in the food web space (Abrantes *et al*., 2014). Therefore, lower values of MNND and SDNND represent greater trophic redundancy, since species have more similar trophic niches. In communities with similar MNND (mean distance) values, lower SDNND represents higher trophic redundancy. A Bayesian approach to these metrics was performed with the SIAR package in R (Stable Isotope Analysis in R; Jackson *et al*., 2011; Parnell and Jackson, 2008), which allows comparing communities containing different sample sizes. The method also allows propagating sample error on the estimates of the means of community components to provide measures of uncertainty surrounding the metrics, allowing robust statistical comparisons among communities. Standard ellipse areas (SEA) were also calculated with the SIBER package (Stable Isotope Bayesian Ellipses in R; Jackson *et al*., 2011) to quantify the isotopic niche of each community. This Bayesian standard ellipse is less sensitive to low sample size and extreme values than the total area proposed by Layman *et al*. (2007b). Therefore, it is a more robust approach for comparisons between communities.

Statistical analyses

All statistical analyses were performed using R software, version 3.6.0. (R Core Team, 2019). We conducted Linear Models with the factors Period (Before and After), Reach (Control and Impact), Season (Autumn, Winter and Spring) and their interaction as sources of variation for the stock of basal resources and maximum food chain length and trophic position of *P. bigerri* and *S. trutta* to assess the effects of the effluent (Period:Reach, Period:Reach:Season). The same sources of variation were used for $\delta^{13}C$ and $\delta^{15}N$ of the entire community and each functional group (basal resources, primary consumers, omnivores, predatory invertebrates, and fish) in Linear-Mixed Effects Models (function lme, in R package nlme (Pinheiro *et al*., 2020)) including also Taxa as random factor. Variance components of Mixed Effects Models were estimated through restricted maximum likelihood and p values estimated by means of likelihood ratio tests (Pinheiro and Bates, 2006).

To test for the effect of the effluent on diet contribution analyses and the iso-space metrics, we used Generalised Linear Models (GLMs) on the posterior estimates of the Bayesian models, since including these variables into the Bayesian models caused a lack of convergence. We included 9000 posterior estimates on diet contribution analyses and 4000 posterior estimates in the iso-space metrics analyses for each variable and community. Different numbers of posterior estimates were included in GLMs because settings to avoid convergence problems in Bayesian models differed. Posterior estimates related to diet contribution analyses were adjusted to a binomial distribution (link: logit) and estimates related to community iso-space metrics followed Gaussian distribution (link: identity) (Zuur *et al*., 2009). Different GLMs were built for each variable using Period, Reach, Season and their interactions as sources of variation (Table S3 and S4): null model, three models with a single source of variation, three models with two sources of variation, three models with two sources of variation and their interaction term, three models with three sources of variation and one double interaction term, two models with the three sources of variation and two double interaction terms, a model with the three sources of variations and the three double interaction terms and the maximal model, which also included the triple interaction term. As the sample size was large, Bayesian Information Criterion (BIC) was used to penalize size and select the best explanatory model in each case (Brewer *et al*., 2016). Model selection was made with the ‘modelsel’ function of the MuMIn package (Barton, 2020). Due to the large amount of posterior estimates, violin plots were used instead of boxplots or dot plots to show the distribution of the results whenever Bayesian modelling was applied. Separate models for each season were computed when the triple Period:Reach:Season interactions were significant or when full models were preferred. Period:Reach coefficients for these separate models are reported in the results section and in the figures to show the effect size associated to each season.

Results

Stock of basal food resources and their contribution to the diet of primary consumers

Coarse detritus stock differed between periods, reaches and seasons (Fig. 2 a, Table 1): its abundance was higher during the After period ($F_{1,96}$ = 4.46, p = 0.037), in the Impact reach compared to Control ($F_{1,96}$ = 12.12, p = 0.001), and highest in Autumn compared to Winter and Spring ($F_{2,96}$ = 34.05, $p < 0.001$). Biofilm stock also differed between periods ($F_{1,48}$ = 30.01, $p < 0.001$, Fig. 2 b , Table 1) and seasons, with higher abundances in Autumn ($F_{2,48}$ = 18.19, $p < 0.001$). Moreover, the addition of the effluent significantly increased its abundance (Period:Reach: $F_{1,48}$ = 7.17, p = 0.010), with different effects depending on the season (Period:Reach:Season: $F_{2,48}$ = 3.83, p = 0.029), mainly increasing in Autumn and Winter (coeff. = 0.44 and 0.63 respectively for models built separately).

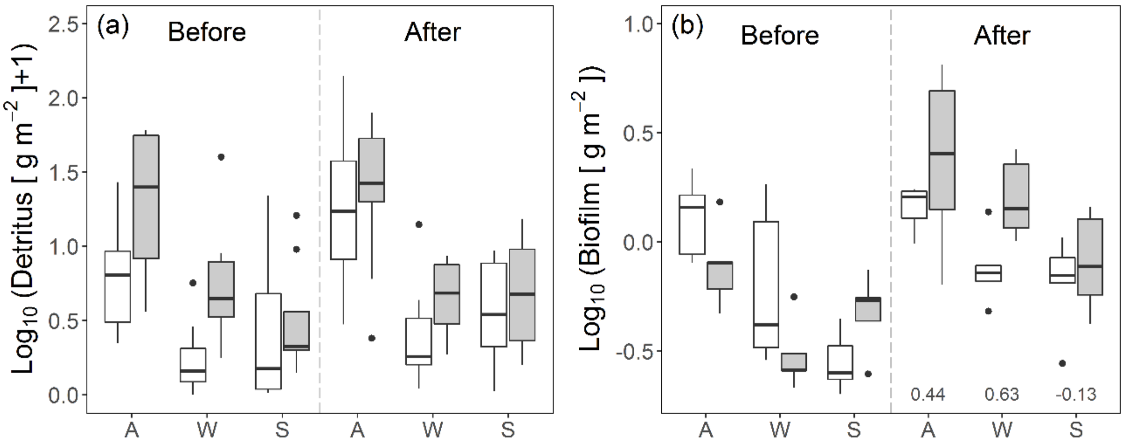


Fig. 2. Abundance of detritus and biofilm in the studied reaches Before and After the addition of the effluent during Autumn (A), Winter (W) and Spring (S). The box plots show the median, the interquartile range and the tails of the distribution, and dots represent outliers. Control reaches are shown in white and Impact reaches in grey. Coefficients for the Period:Reach interaction obtained from separate models for each season are shown when the interaction Period:Reach:Season was significant.

Table 1. Linear model results comparing stock of basal resources to assess the effects of effluent addition (Period:Reach, Period:Reach:Season). Values in bold indicate statistical significance ($p < 0.05$). Coefficients are shown for significant responses and consider Before Period, Control Reach and Autumn Season as reference in all cases. A is After, I is Impact, and W and S refer to Winter and Spring respectively.

	Coarse detritus (N=108)				Biofilm (N=60)			
	F	p	Coeff.		F	p	Coeff.	
Period	4.46	**0.037**	0.48 (A)		30.01	**<0.001**	0.04 (A)	
Reach	12.22	**0.001**	0.54 (I)		0.88	0.353		
Season	34.05	**<0.001**	-0.55 (W)	-0.39 (S)	18.19	**<0.001**	-0.32 (W)	-0.66 (S)
Period:Reach	2.07	0.153			7.14	**0.010**	0.44 (A:I)	
Period:Season	0.67	0.512			0.53	0.589		
Reach:Season	1.30	0.279			0.83	0.442		
Period:Reach:Season	0.76	0.472			3.83	**0.029**	0.2 (A:I:W)	-0.57 (A:I:S)

Coarse detritus was generally the main contributor to the diet of primary consumers, followed by autochthonous resources (Fig. 3). Fine detritus contribution was not affected by the addition of the effluent (Fig. 3 b, Table 2, S3 and S5). However, contributions of coarse detritus and autochthonous resources were altered and also differed depending on the season (Table 2). The contribution of autochthonous resources increased with the addition of the effluent in Autumn and Winter (Fig. 3 c, Table 2, S3; coeff. Autumn = 0.44, coeff. Winter = 0.54 for models built separately), but it decreased in Spring (coeff. = -0.41), whereas coarse detritus followed the opposite trend (Fig. 3 a, Table 2, S5; coeff. Autumn= -0.54, coeff. Winter = -0.30, coeff. Spring = 0.49).

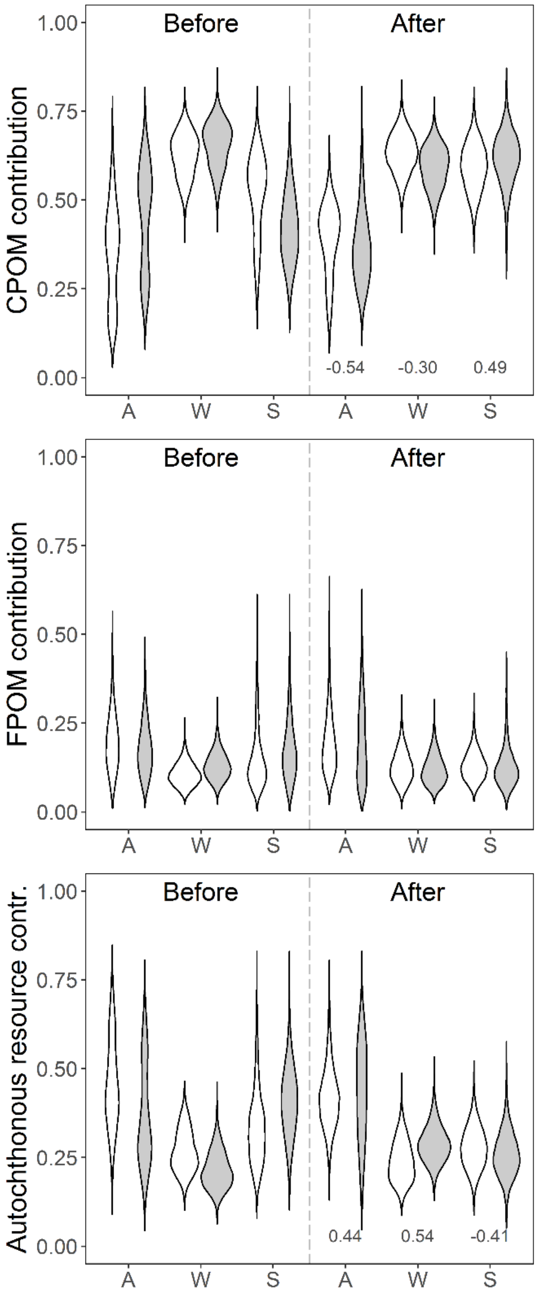


Fig. 3. Bayesian posterior estimates showing the contribution of (a) coarse detritus (CPOM), (b) fine detritus (FPOM) and (c) autochthonous resources (biofilm, filamentous green algae, and bryophytes) to the diets of consumers (white for control; grey for diverted) in Autumn (A), Winter (W) and Spring (S) Before and After the start of the effluent addition. Coefficients for the Period:Reach interaction obtained from separate models for each season are shown when full models were the preferred models.

Table 2. Preferred models to explain the effect of the effluent addition on the contribution of basal resource (coarse detritus, fine detritus and autochthonous resources) to primary consumers. Degrees of freedom (df), log-likelihood ratios (logLik) and Bayesian Information Criterion (BIC) are given. Coefficients of preferred models are shown when $p < 0.05$. Before Period and the Control Reach and Autumn Season are considered as reference in all cases. A refers to After and I to Impact, W to Winter and S to Spring.

	CPOM contr.		FPOM contr.	Autochthonous resource contr.	
Model	Full model		Null model	Full model	
df	12		1	12	
logLik	-59242.56		-17906.6	-48142.48	
BIC	118624.2		35824.8	96424	
Period	<0.001	0.18 (A)		<0.001	-0.16 (A)
Reach	0.803			0.112	
Season	<0.001	1.15 (W), 0.7 (S)		<0.001	-0.84 (W), -0.53 (S)
Period:Reach	<0.001	-0.54 (A:I)		<0.001	0.44 (A:I)
Period:Season	<0.001	-0.15 (A:W), 0.12 (A:S)		<0.001	0.01 (A:W), -0.1 (A:S)
Reach:Season	<0.001	-0.33 (I:W), -0.85 (I:S)		<0.001	0.09 (I:W), 0.7 (I:S)
Period:Reach:Season	<0.001	0.24 (A.I:W), 1.03 (A:I:S)		<0.001	0.09 (A.I:W), -0.86 (A:I:S)

Stable isotope signatures and food chain length

On the one hand, the $\delta^{15}N$ signatures significantly differed between periods, reaches and seasons (Fig. 4 a, Table 3): signatures were higher in the After period ($F_{1,1921} = 95.17$, $p < 0.001$) and in the Impact reach compared to Control ($F_{1,1921} = 294.63$, $p < 0.001$). Autumn also showed the greatest $\delta^{15}N$ values compared to Winter and Spring ($F_{2,1921} = 258.00$, $p < 0.001$). Moreover, the $\delta^{15}N$ ratio increased significantly with the effluent addition (Period:Reach: $F_{1,1921} = 54.15$, $p < 0.001$, coeff. = 1.3, Fig. 4 a, Table 3) with similar effects across seasons (i.e. non-significant triple interaction). All the groups and basal food resources got their $\delta^{15}N$ signature enriched with the effluent (Period:Reach interaction, Fig. S1 a-d, Table S6, S7), except for coarse detritus that did not vary (Table S7) and fish, who showed a depletion in presence of the effluent (Figure S1 e; Table S6). Nonetheless, when considering each season separately, although Autumn showed a decrease in $\delta^{15}N$, Winter and Spring increased with the effluent (Fig. S1 e). However, maximum food chain length and trophic position of the two most frequent species, which are related to $\delta^{15}N$, did not differ in presence of the effluent ($F_{1,34} = 1.03$, $p = 0.317$; $F_{1,46} = 0.54$, $p = 0.466$ and $F_{1,34} = 0.02$, $p = 0.876$; Fig. 5; Table 4). Maximum food chain length was larger in Impact reaches ($F_{1,34} = 5.08$, $p = 0.031$; Fig. 5, Table 4). *P. bigerri* showed the highest trophic position in most of the sampling occasions (Table S2), and showed a higher trophic position in the Impact compared to Control reach ($F_{1,46} = 11.19$, $p = 0.002$; Fig. 5, Table 4). *S. trutta* showed similar values between reaches in each sampling occasion (Fig. 5, Table S2). On the other hand, $\delta^{13}C$ also differed between reaches and seasons ($F_{1,1921} = 83.90$, $p < 0.001$ and $F_{2,1921} = 92.02$, $p < 0.001$ respectively; Fig. 4 b, Table 3), but again, effluent did not affect it (Period:Reach: $F_{1,1921} = 0.39$, $p = 0.534$).

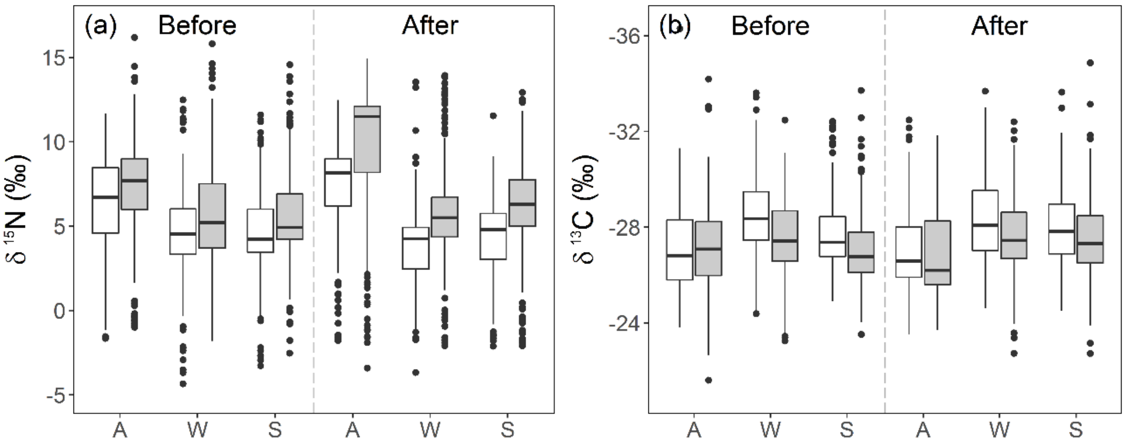


Fig. 4. (a) Nitrogen stable isotope ratios ($\delta^{15}N$, ‰) and (b) Carbon stable isotope ratios ($\delta^{13}C$, ‰) of the entire community in the studied reaches (white for control; grey for diverted) in Autumn (A), Winter (W) and Spring (S) during the Before and After periods. The box plots show the median, the interquartile range, and the tails of the distribution, and dots represent outliers.

Table 3. Linear mixed model results of stable isotope ratios for the entire community with Period, Reach and Season as fixed factors and sample as random. Values in bold indicate significant differences between factors ($p < 0.05$). Coefficients are shown for significant responses and consider Before Period, Control Reach and Autumn Season as reference in all cases. A is After, I is Impact, and W and S refer to Winter and Spring respectively.

	$\delta^{15}N$				$\delta^{13}C$			
	F	p	Coeff.		F	p	Coeff.	
Period	95.17	**<0.001**	1.18 (A)		3.34	0.068		
Reach	294.63	**<0.001**	0.77 (I)		83.90	**<0.001**	-0.02 (I)	
Season	258.00	**<0.001**	-0.9 (W)	-1.1 (S)	92.02	**<0.001**	-1.19 (W)	-0.28 (S)
Period:Reach	54.15	**<0.001**	1.3 (A:I)		0.39	0.534		
Period:Season	68.77	**<0.001**	-1.73 (A:W)	-1.27 (A:S)	21.39	**<0.001**	0.2 (A:W)	-0.57(A:S)
Reach:Season	2.44	0.087			9.94	**<0.001**	0.76 (I:W)	0.54 (A:S)
Period:Reach:Season	1.94	0.144			2.69	0.068		

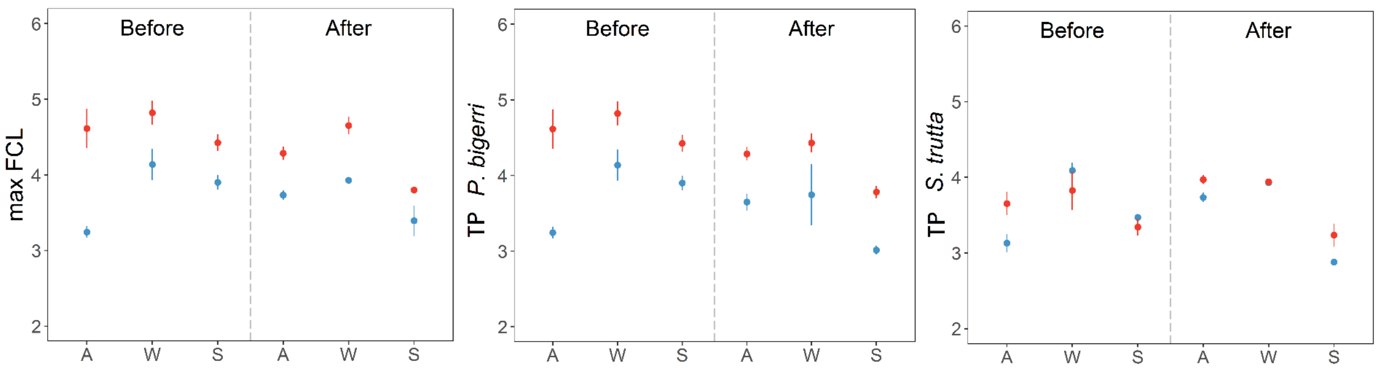


Fig. 5. Maximum food chain length (max FCL) and trophic position (TP) of the two most frequent species (*P. bigerri* and *S. trutta*) in the studied reaches (blue for control; red for impact) in Autumn (A), Winter (W) and Spring (S) during the Before and After periods.

Table 4. Linear model results comparing maximum FCL and TP of the two most frequent species to assess the effects of effluent addition (Period:Reach, Period:Reach:Season). Values in bold indicate statistical significance (p < 0.05 Coefficients are shown for significant responses and consider Before Period, Control Reach and Autumn Season as reference in all cases. A is After, I is Impact, and W and S refer to Winter and Spring respectively.

	max FCL				TP$_{\textit{P. bigerri}}$				TP$_{\textit{S. trutta}}$			
	F	p	Coeff.		F	p	Coeff.		F	p	Coeff.	
Period	5.08	**0.031**	0.49 (A)		11.19	**0.002**	0.41 (A)		0.05	0.819		
Reach	65.9	**<0.001**	1.37 (I)		56.83	**<0.001**	1.37 (I)		3.55	0.068		
Season	7.09	**0.003**	0.89 (W)	0.66 (S)	8.45	**0.001**	0.89 (W)	0.66 (S)	16.96	**<0.001**	0.96 (W)	0.34 (S)
Period:Reach	1.03	0.317			0.54	0.466			0.02	0.876		
Period:Season	3.05	0.060			5.07	**0.010**	-0.8 (A:W)	-1.29 (A:S)	5.93	**0.006**	-0.76 (A:W)	-1.20 (A:S)
Reach:Season	3.12	0.057			1.31	0.280			2.30	0.116		
Period:Reach:Season	1.58	0.221			2.01	0.146			1.62	0.212		

Trophic structure

Trophic diversity and redundancy were best explained by full models, i.e. models containing Period:Reach:Season interaction (Fig. 6, Table 5, S4 and S8). The addition of the effluent reduced trophic diversity (CD) in Autumn and Winter (Fig. 6 a, coeff. = -0.90 and -0.24 respectively for models built separately), whereas it increased in Spring (Fig. 6 a, coeff.= 0.13). The effluent increased trophic redundancy (i.e. lower MNND) in Autumn and Winter with the effluent (Fig. 6 c, Table 5, coeff. = -0.27 and -0.57 for models built separately), whereas it reduced it on Spring (Fig. 6 c,Table 5, coeff. 0.18). Nevertheless, SDNND in Winter was higher with the addition of the effluent (Table S8, coeff. = 0.14), representing a more heterogeneous spacing of taxa. In Spring, trophic diversity decreased in presence of the effluent (higher MNND; coeff. = 0.18). Finally, community niche space (SEA), decreased in Autumn and Spring with the addition of effluent (coeff. = -0.42 and -1.03 respectively for models built separately), but increased in Winter (coeff. = -1.03).

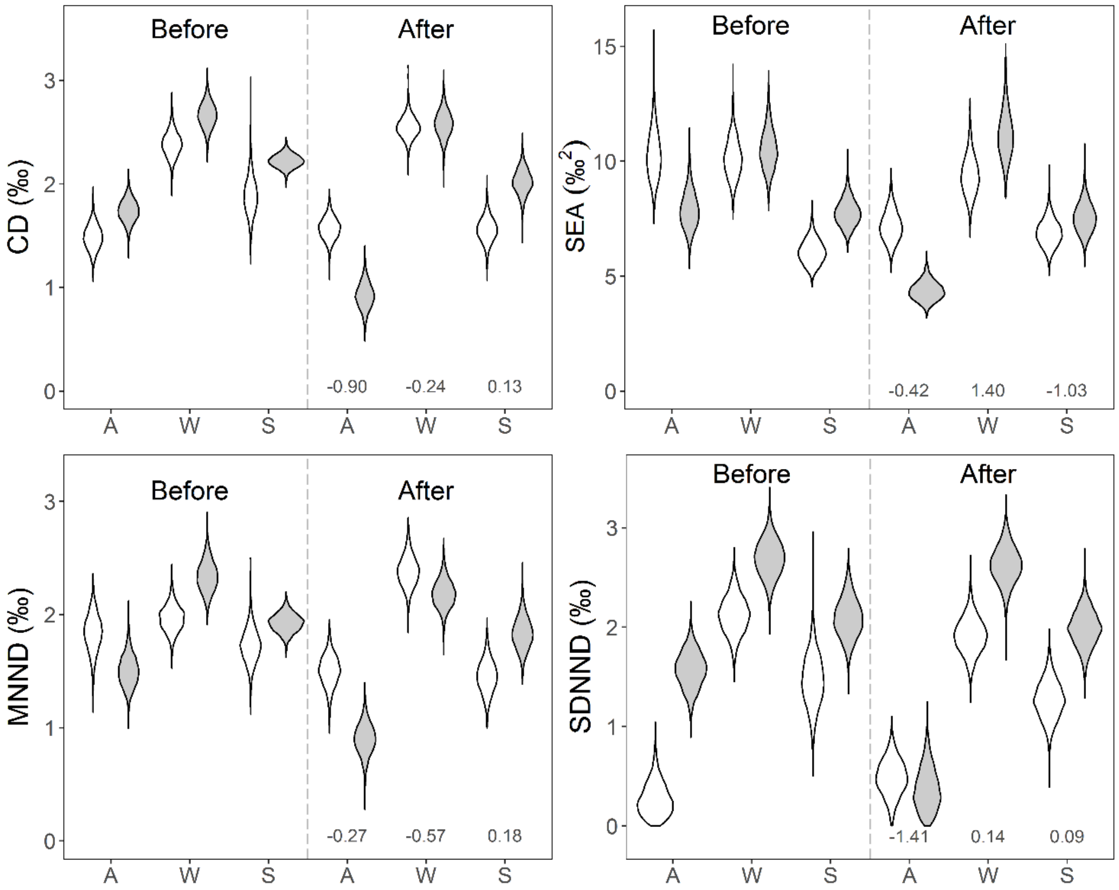


Fig. 6. Bayesian posterior estimates of community wide metrics of the iso-space for consumers: (a) distance to centroid (CD), (b) standard ellipses area (SEA), (c) mean nearest neighbour distance (MNND) and (d) standard deviation of nearest neighbour distance (SDNND). These metrics provide information about trophic diversity, community niche space and redundancy of the eight consumer communities (white for control; grey for diverted) in Autumn (A), Winter (W) and Spring (S) Before and After the start of the effluent addition. Coefficients for the Period:Reach interaction obtained from separate models for each season are shown when full models were the preferred models.

Table 5. Preferred models to explain the effect of the effluent addition on the iso-space metrics of the consumers (invertebrates and fish). Degrees of freedom (df), log-likelihood ratios (logLik) and Bayesian Information Criterion (BIC) are given. Coefficients of preferred models are shown when $p < 0.05$. Before Period and the Control Reach and Autumn Season are considered as reference in all cases. A refers to After and I to Impact, W to Winter and S to Spring.

	CD		SEA		MNND		SDNND	
Model	Full model		Full model		Full model		Full model	
df	13		13		13		13	
logLik	33254.16		-55544.76		27785.93		9407	
BIC	-66368.2		111229.7		-55431.7		-18673.9	
Period	<0.001	0.09 (A)	<0.001	-3.11 (A)	<0.001	-0.34 (A)	<0.001	0.21 (A)
Reach	<0.001	0.26 (I)	<0.001	-2.41 (I)	<0.001	-0.33 (I)	<0.001	1.3 (I)
Season	<0.001	0.9 (W), 0.4 (S)	<0.001	-0.16 (W) , -4.25 (S)	<0.001	0.13 (W) , -0.11 (S)	<0.001	1.85 (W) , 1.19 (S)
Period:Reach	<0.001	-0.9 (A:I)	0.181		<0.001	-0.27 (A:I)	<0.001	-1.41 (A:I)
Period:Season	<0.001	0.08 (A:W) , -0.41 (A:S)	<0.001	2.35 (A:W) , 3.94 (A:S)	<0.001	0.76 (A:W) , 0.06 (A:S)	<0.001	-0.42 (A:W) , -0.41 (A:S)
Reach:Season	<0.001	-0.41 (I:W) , 0.08 (I:S)	<0.001	2.79 (I:W) , 4.11 (I:S)	<0.001	0.71 (I:W) , 0.53 (I:S)	<0.001	-0.73 (I:W) , -0.67 (I:S)
Period:Reach:Season	<0.001	0.66 (A.I:W) , 1.04 (A:I:S)	<0.001	1.82 (A.I:W) , -0.61 (A:I:S)	<0.001	-0.31 (A.I:W) , 0.45 (A:I:S)	<0.001	1.55 (A.I:W) , 1.5 (A:I:S)

Discussion

Waste water treatment plant (WWTP) effluents are one of the main pollution sources for freshwater ecosystems (Rice and Westerhoff, 2017). Depending on effluent composition, their dilution and the characteristics of the receiving systems, their effects can strongly vary. In the whole-ecosystem manipulation experiment presented here, it is important to note that we are assessing the ecological consequences of properly treated and highly diluted effluents on food web complexity.

The effluent promoted biofilm biomass and increased the contribution of autochthonous resources to primary consumers diets

Overall, mixing models pointed coarse detritus of terrestrial origin as the main basal resource in our study site. It has been widely accepted that leaf litter is the major allochthonous input in forested headwater streams, and thus, the main energy and nutrient source for heterotrophic aquatic communities (Vannote *et al*., 1980; Graça *et al*., 2001; Zhang *et al*., 2019). However, with the addition of the effluent, a trophic shift was observed, since coarse detritus contribution decreased as the contribution of autochthonous resources was promoted. This drop of the contribution of detritus was evident in autumn (when the stream concentration of the effluent was highest) and in winter, but the opposite was true for spring. There is abundant evidence for the increase in detritus use as a consequence of nutrient enrichment (Gulis *et al*., 2006; Woodward *et al*., 2012). In the multifunctional assessment of the effect of this particular WWTP at the same manipulative experiment (Pereda *et al*., 2020) also demonstrated a significant increment of the overall decomposition, which was attributed mainly to the action of detritivores. The higher organic processing associated to a lower contribution of detritus to primary consumers highlights the mismatch between the effect of the manipulation of resources and its assimilation by consumers.

Biofilm biomass increase with nutrient concentrations (Francoeur, 2001; Ribot *et al*., 2015; Dodds and Smith, 2016) and also under sewage inputs (Pereda *et al*., 2019, 2021), where pollution-resistant taxa form highly functional microbial communities (Rosi *et al*., 2018). In our study, although coarse detritus dominated the stock of basal resources and it was higher than biofilm stock, biofilm biomass significantly increased with the effluent, increasing its relative availability over the availability of coarse detritus. The response of biofilm stock to effluent addition was paralleled by a drift from brown to green pathway, since contrarily to what happened to detritus, higher contribution of autochthonous resources was also linked to the addition of the effluent. Thus, the relative increase in autochthonous production stimulated herbivory in the community. Similarly, Baumgartner and Robinson (2017) also assessed changes in dietary composition of primary consumers, who switched their mainly detritivorous diet upstream from a WWTP towards herbivory in downstream reaches. In a similar system where detritus was also the most abundant basal resource, Bumpers *et al*. (2017) observed a shift from brown to green food-web pathways with nutrient enrichment. Additionally, the response of the stock of biofilm to the effluent addition differed among seasons.

Besides nutrient availability, seasonal characteristics such as shading and hydrologic disturbances also determine primary production (Bernhardt *et al*., 2018). Alberts *et al*. (2018), for instance, reported higher biofilm biomass in urban streams where nutrient availability was higher than in reference streams, but highlighted the impact of the light limitation driven by the riparian canopy, which produced a decline in biofilm biomass during summer. When assessing the recovery of biofilm communities under sewage inputs after a flooding event, Merbt *et al*. (2011) also highlighted the importance of lighting, as the recovery was faster for biofilms directly exposed to light. In our study, biofilm accrual increased with the addition of the effluent, especially in Autumn and Winter. The concentration of the effluent in our manipulated reach was the highest just before the autumn sampling. During this period, the effluent contained higher total nitrogen concentration than in the other seasons. However, it was in winter when biofilm biomass increased the most, reflecting a legacy effect of the effluent and denoting that despite being temporally variable, biofilm communities undergo a successive accumulation (Merbt *et al*., 2011). In Spring, however, although effluent contribution was similar to that in Winter, biofilm stock was responding negatively to the effluent, which was mimicked again by the contribution of autochthonous resources to herbivory. This reduction in biofilm biomass could be explained by the press perturbation exerted in the impacted site with the addition of the effluent. Biofilm communities in control and impact reaches might have been affected by the heavy rains that increased stream flow and shear forces, reducing biofilm biomass. The recovering community in the impact reach, apart from receiving nutrients with the effluent was also receiving toxic compounds, which could have a destabilizing effect on the community.

Community's $\delta^{15}N$ signature increased but changes were not transferred to maximum food chain length

Treated effluents get isotopically enriched or depleted in $\delta^{15}N$ depending on the treatments they receive. Secondary and tertiary treated effluents are isotopically enriched in the heavier ^{15}N isotope due to the enzymatic preference of bacteria for the lighter ^{14}N during the denitrification process (Finlay and Kendall; Morrissey *et al*., 2013), whereas non treated or primary treated sewages get depleted (Daskin *et al*., 2008; di Lascio *et al*., 2013). Typically, the faster loss of ^{14}N than ^{15}N in metabolism and excretion induces higher $\delta^{15}N$ values in consumers than their immediate resources, with estimated increases of 2.2-3.4 ‰ (Vander Zanden and Rasmussen, 2001; Fry, 2006), and increases in $\delta^{15}N$ at the base of the food web due to environmental enrichment should also be transferred along the food web. In our study, the increased $\delta^{15}N$ signature of the tertiary treated effluent was reflected in the entire community, but the increased signature was not transferred equally into the different functional groups. The stock of basal resources showed a general increase in $\delta^{15}N$ with the effluent, but when assessing effluent enrichment of $\delta^{15}N$ in each group, we could observe the increase occurred in biofilm, in the autochthonous resource group and fine detritus, but coarse detritus remained unchanged. This results go in line with observations made in other studies assessing nutrient enrichment or pollution effects, where autochthonous resources such as biofilm readily assimilate the enriched N but the increase is not so evident in terrestrial detritus (Pastor *et al*., 2014; Baumgartner and Robinson, 2017; de Guzman *et al*., 2021). As reported in other studies assessing effects of effluent inputs (Gücker *et al*., 2011; Morrissey *et al*., 2013; Robinson *et al*., 2016; Baumgartner and Robinson, 2017), primary consumers, omnivores and carnivores also got enriched in the present experiment. However, with the addition of the effluent fish got their $\delta^{15}N$ signature slightly depleted during Autumn, although it was slightly enriched in Winter and Spring. Robinson *et al*. (2016) reported contrasting results in two fish species of a small stream receiving a high quality effluent, where while one species got enriched following the same trend of the invertebrate community, the other species got depleted in ^{15}N. As a plausible explanation they suggested the depleted species could have been feeding on the least enriched invertebrates of the reach or outside the effluent plume. Due to the high mobility of fishes, the small size of the manipulated reach in our study and the facility of accessing the larger nearby river, this small changes could suggest that fish of our study could also have been feeding out of the monitored reaches. However, the increase in $\delta^{15}N$ during Winter and Spring, seasons when the contribution of the effluent was lower, suggests a lag of the enrichment of tissues by $\delta^{15}N$ with the effluent, which matches the longer turnover rate in fish tissues than in invertebrates (Vander Zanden *et al*., 2015).

Following the productivity hypothesis (Pimm, 1982) and considering the low toxicity of the effluent microbial performance and detritivore growth (Solagaistua *et al*., 2018), we expected the higher contribution of autochthonous resources of higher nutritional quality to reorganize food webs, lengthening food chains due to the larger overall nutrient availability for secondary production (Lau *et al*., 2009; Junker and Cross, 2014). However, we did not detect any variation in the maximum food chain length with the effluent. When assessing food chain length with invertebrates, Price *et al*. (2019) did not observe longer food chains at urban streams either, suggesting that the influence of basal productivity could be weaker than the influence of other factors such as ecosystem size in these small ecosystems (Ward and McCann, 2017). In addition, in our study, maximum food chain length was systematically larger in the impact reach than in the control one. Despite being herbivorous, *P. bigerri* was the species with the highest trophic position in most of the sampling campaigns. The study site characteristics could facilitate the access of individuals from the impact reach to feed into the larger Deba River, compared to individuals from the control reach, which could have upstream, more oligotrophic, reaches more accessible for feeding. The commonly larger trophic position of this species leads us consider a non-selective feeding habit of these individuals, who might also be feeding on small but $\delta^{15}N$ enriched preys. The lack of differences between trophic positions of *S. trutta* between reaches could be driven by the bigger mobility of this species together with the tendency of this species of eating terrestrial food resources (Erős *et al*., 2012) which could also be more depleted in ^{15}N than the aquatic preys, thus explaining both the lower trophic position and the lack of response to the effluent addition. Moreover, these differences of trophic position between *S. trutta* and *P. bigerri* could also suggest that the former relies more on the less $\delta^{15}N$ enriched brown pathway and the latter on the more reactive green pathway. This idea is supported by the preferences of these two fish species for aquatic macroinvertebrates: Gammarids, Ephemeroptera and Trichoptera for *S. trutta* (Oscoz *et al*., 2000) and Chironomids for *P. bigerri* (Leunda *et al*., 2017).

Finally, we expected a rearrangement of the niche space of consumers towards more diverse and less overlapped niches due to the increase in nutrient availability and the relatively higher biofilm biomass. Several studies have linked moderate pollution and the consequently larger biofilm availability to a greater isotopic variability (Parreira de Castro *et al*., 2016; García *et al*., 2017) due to the increase in isotopic diversity of basal food resources. Stronger pollution levels however, can cause a reduction in resource diversity by decreasing isotopic variability and leading to a narrower isotopic variation of consumers (García *et al*., 2017). Just downstream from an effluent outfall de Carvalho *et al*. (2020) observed the highest trophic diversity of fish assemblages in their study, suggesting either that the available resources had a more variable isotopic signatures, that there was an intra and inter-specific variation in turnover rates of fish tissues, or even that the species of the most polluted sites were more generalist than in the other study sites, increasing the variability of the niches. Still, there are many studies suggesting a decrease in trophic redundancy in food webs at urban sites (di Lascio *et al*., 2013; Price *et al*., 2019), a typically contrasting variable with trophic diversity, due to the abundant but poorly diversified resources or even because of local extinctions. Contrary to what we expected, we observed a decrease in trophic diversity and an increase in redundancy in the seasons when the contributions of autochthonous resources increased. The effluent used during the present study showed low toxicity (Solagaistua *et al*., 2018), and in general, the macroinvertebrate community did not suffer a reduction in diversity nor an alteration in abundance (González *et al*., *In prep.*), although drops in diversity and abundance of sensitive taxa were observed. Within the five most abundant taxa, chironomids and oligochaetes significantly increased, whereas *Baetis* and *Potamopyrgus* decreased (González *et al*., *In prep.*). The fish assemblage was also similar in each sampling occasion. However, when considering the period with the highest effluent contribution, a decrease in local diversity was observed (Chapter 4).Thus, the observed reduction in diversity could be responsible of the reported decrease in trophic diversity.

Conclusions

The present whole-ecosystem manipulation experiment reveals changes in food web complexity even when dealing with well treated and highly diluted effluents. Even small impacts can modify ecosystem productivity, affecting green and brown food web pathways, which might end up altering food web composition and associated ecosystem functioning. Our experiment suggests that current methods for treating polluted waters might be insufficient to preserve natural properties of food webs. This would be especially relevant when aiming conservation of highly sensitive aquatic ecosystems.

Acknowledgements

This research was supported by the EU Seventh Framework Programme (GLOBAQUA; 603629-ENV-2013-6.2.1). Authors also acknowledge the financial support from the Basque Government (Consolidated Research Group IT951-16) and the pre-doctoral fellowship from the Basque Government (I. de Guzman). D. von Schiller is a Serra Húnter Fellow. J. M. Montoya is funded by the FRAGCLIM Consolidator Grant (number 726176) from the European Research Council under the EU Horizon 2020 Research and Innovation Program and by the "Laboratoires d’Excellences (LABEX)" TULIP (ANR-10-LABX-41). Authors are especially grateful to Libe Solagaistua, Olatz Pereda, Vicki Perez, Miren Atristain, Miren Barrado, Mikel Buceta and Ines Locker for their assistance during field campaigns and laboratory analyses, and to the staff of the Apraitz WWTP for continuous support during the experiment.

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Chapter 4

Treated and highly diluted wastewater impacts diversity and energy fluxes of freshwater food webs

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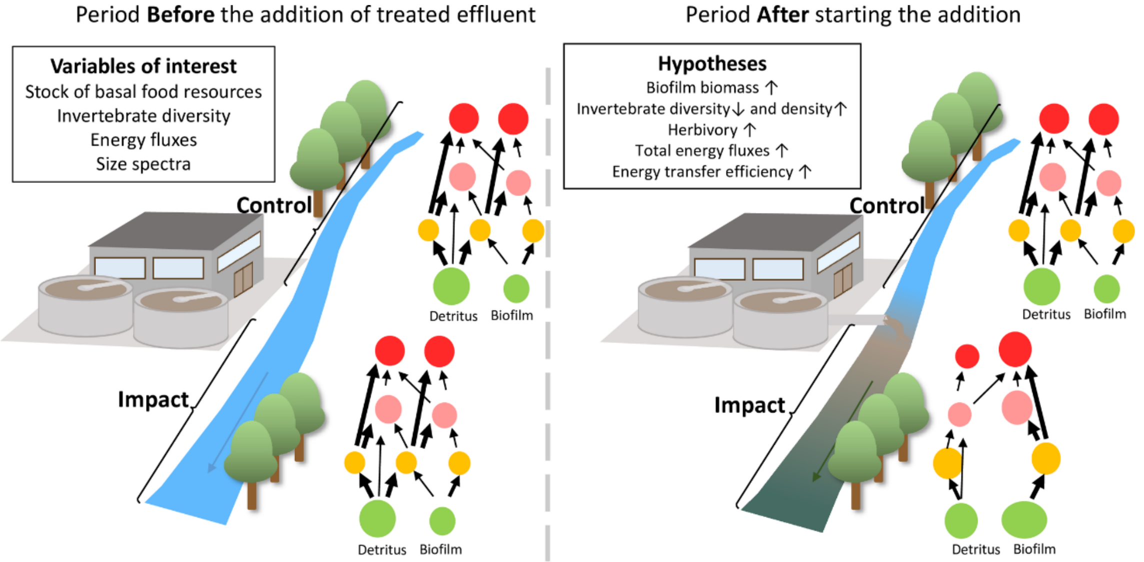
Journal of Animal Ecology, Submitted

Abstract

Wastewater Treatment Plants (WWTPs) have greatly improved water quality globally. However, treated effluents still contain a complex cocktail of pollutants whose environmental effects might go unnoticed, masked by additional stressors in the receiving waters or by spatiotemporal variability. We conducted a BACI (Before-After/Control-Impact) ecosystem manipulation experiment, where we diverted part of the effluent of a large tertiary WWTP into a small, unpolluted stream to assess the effects of a well-treated and highly diluted effluent on riverine diversity and food web dynamics. We sampled basal food resources, benthic invertebrates and fish to search for changes on the structure and energy transfer of the food web with the effluent. Although effluent toxicity was low, it reduced diversity, increased primary production and herbivory, and reduced energy fluxes associated to terrestrial inputs. Altogether, the effluent decreased total energy fluxes in stream food webs, showing that treated wastewater can lead to important ecosystem-level changes, affecting the structure and functioning of stream communities even at high dilution rates. The present study shows that current procedures to treat wastewater can still affect freshwater ecosystems and highlights the need for further efforts to treat polluted waters to conserve aquatic food webs.

Key words: *BACI experiment, community size-spectra, diversity, ecosystem-level manipulation, energy fluxes, freshwater food webs, pollution, treated wastewaters*



Introduction

Population growth and socio-economic development in the last decades has led to a global increase of urbanization, with over 55% of the world population living in urban areas (United Nations 2019). To reduce anthropogenic pollution, Wastewater Treatment Plants (WWTP) have been widely implemented. Although they have greatly reduced the amount of contaminants reaching aquatic ecosystems (Vaughan & Ormerod 2012; Brion *et al.* 2015), treated sewage still consists of a complex mixture of pollutants, nutrients, and pathogens (Pascual-Benito *et al.* 2020). Some of these compounds are simply toxic (Patel *et al.* 2020; Vasilachi *et al.* 2021), whereas others, such as nutrients, subsidize biological activity, although they can also become hazardous above a certain concentration (Carey & Migliaccio 2009; Wang *et al.* 2019). Thus, the effects of effluents on streams strongly depend on effluent composition and dilution rate, being strongest when poorly diluted in the receiving water mass (Carey & Migliaccio 2009; Arenas-Sánchez *et al.* 2016).

By releasing resources such as nutrients and organic matter, WWTP effluents can stimulate ecosystem productivity. In small forested streams where primary production is limited by nutrients (Tank & Dodds 2003; Elser *et al.* 2007) and canopy cover (Bernhardt *et al.* 2018), detritus, mainly in the form of leaf litter, is the main food resource (Vannote *et al*., 1980; Zhang *et al*., 2019). This detrital OM, colonized by microbes, becomes thus the base of brown food webs (Marks 2019). However, algae, albeit rare, also play key roles in these ecosystems (e.g. Brett *et al*., 2017), as they provide a high quality food resources with important consequences for both green and brown pathways of food webs (Marcarelli *et al.* 2011; Crenier *et al.* 2017) that can stimulate or inhibit organic matter processing (Halvorson *et al.* 2016, 2019).

Therefore, WWTP effluents could trigger bottom-up trophic cascades, affecting the composition and structure of communities and food webs, and ultimately altering ecosystem functioning (Woodward *et al.* 2005; Thompson *et al.* 2012). A useful way to infer information on these changes is to analyse the distribution of individual body sizes in the community(i.e. community size-spectra), as it informs of how energy is shared and transported along the food web (White *et al.* 2007). The slope of the abundance-mass scaling relationship on a log scale informs on the trophic transfer efficiency in the community (Trebilco *et al.* 2013), being shallower when the energy is transferred more efficiently towards the higher trophic levels, and thus supports higher abundance of large species (Woodward *et al*. 2005). Changes in energy transfer efficiency can be driven by modifications in the nutrient and resource cycles. For instance, nutrient inputs may reduce the stoichiometric gap between consumers and their resources, which would in turn increase trophic transfer efficiency (Mulder & Elser 2009; Ott *et al.* 2014; Xu *et al.* 2015), whereas toxic compounds may decrease the abundance of an specific size class (Baho *et al.* 2019) or increase the abundance of tolerant taxa (Peralta-Maraver *et al.* 2019). These changes are likely to modify energy fluxes in the food web, altering ecosystem functioning (Mulder *et al.* 2008; Brose *et al.* 2017).

The ecological effects of poorly treated and highly concentrated WWTP effluents are evident. The effects of well-treated and highly diluted effluents, although weaker, could still be significant since even low pressures can result in clear changes in ecosystems (Hillebrand *et al.* 2020). However, these effects can remain unnoticed by common monitoring surveys as receiving water masses are often subject to other co-occurring stressors or because difficulties to isolate the effects of interest from confounding factors inherent to spatiotemporal variability arise. This situation calls for carefully designed experimental studies (Downes *et al.* 2002). We performed a whole-ecosystem manipulation experiment following a BACI (Before-After/Control-Impact) design, in which we diverted part of the effluent of a WWTP to a nearby, unpolluted stream, and assessed its effect on the food web. We studied the effects of the effluent on diversity, on food web structure and on the efficiency of energy fluxes across the food web. Because a previous study showed little toxicity of the effluent for invertebrates and microbes (Solagaistua *et al.* 2018), we did not expect large toxic effects on the food web either. However, we expected the most sensitive taxa to decrease in abundance, with detrimental effects on diversity. We also predicted the effluent addition to promote energy flux through the green food web, increasing herbivory and the dependency of the food web on biofilm. We finally predicted that enhanced nutrient inputs would promote the efficiency of the energy transfer along the food web, leading to shallower slopes of the size spectra (Fig. 1).

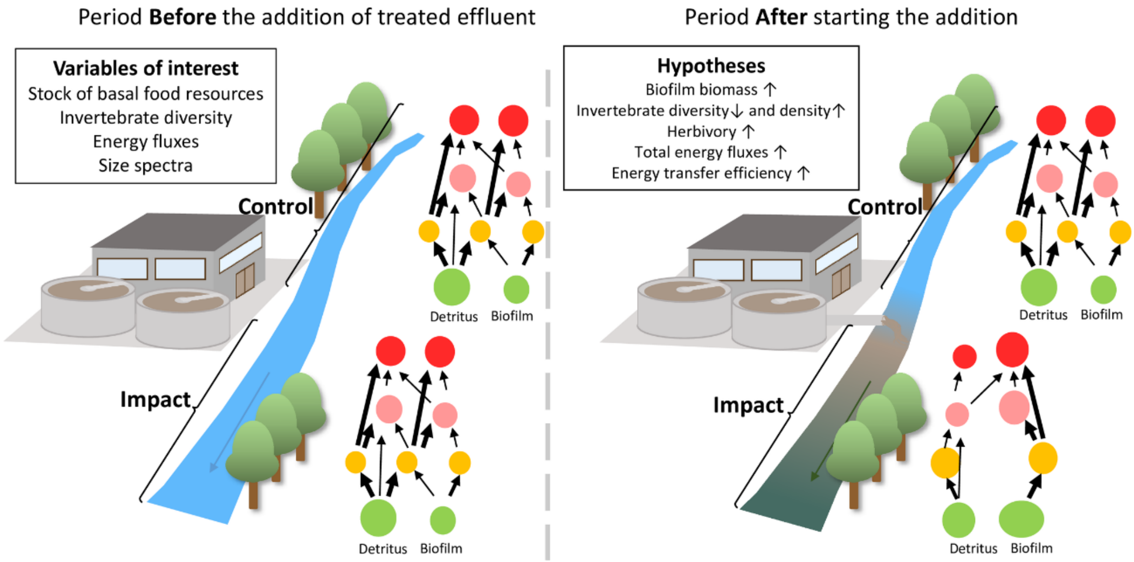


Fig. 1. Schematic drawing of the BACI (Before-After-Control-Impact) experimental design. The Control reach is upstream from the effluent pouring location. The Impact site receives treated effluent during the After period.

Materials and methods

Study site and experimental design

The experiment was conducted in the Apraitz Stream (N Iberian Peninsula, 43°13'41.1"N 2°23'56.3"W), a small unpolluted stream with a mean discharge of 0.12 m^3s^{-1} draining a 7 km^2 catchment over sandstone and shale. In the lowermost 300 m, its riverbed is dominated by bedrock and cobbles. The young riparian forest surrounding it is mainly composed of black alder (*Alnus glutinosa* (L.) Gaertn.), hazel (*Corylus avellana* L.) and ash (*Fraxinus excelsior* L). This reach runs next to the WWTP of Apraitz which releases the treated sewage into the Deba River (mean ± SE discharge during our study 10.9 ± 0.7 m^3s^{-1}, http://gipuzkoa.eus/) through a regularly pulsed discharge (20-40 minutes every 2 h). The WWTP receives the sewage of approximately 90,000 population equivalents from urban and industrial areas, treating on average 29,904 m^3 of wastewater per day in sequential biological reactors (https://www.acciona-agua.com). After mixing the sewage with activated sludge and subjecting it to aerobic and anaerobic processes to reduce the load of organic matter and nitrogen, it receives tertiary treatment (precipitation with ferric sulphate) to reduce phosphorus concentrations.

The experiment followed a BACI design (Fig. 1), which allows controlling both temporal and spatial variability to isolate the effect of interest (Downes *et al.* 2002). We diverted part of the WWTP effluent into the lowermost 150 m of the Apraitz Stream to get a final dilution rate similar to that of the effluent discharged into the Deba River (0.2-4 % and 0.1-9 % of effluent concentration, respectively (Pereda *et al.* 2020)). We studied two 100-m long reaches: a Control reach, upstream from the effluent addition point and an Impact reach just below. Reaches were separated by a 20 m-long buffer reach that was composed by a man-made concrete flat surface that was able to reduce water depth to less than 5 cm and that ended in an overhung waterfall of about 50 cm. Both reaches formed a continuum only on the strongest spates. Although the downstream drift could not be avoided, upstream migration of fish was virtually impossible. For this study, one sampling was conducted at each reach before (September 2016) and after (October 2017) the start of the effluent addition (May 2017), low-flow periods entailing a low dilution of the effluent during the after period. The effluent release changed significantly water physico-chemical characteristics at the impact reach (Pereda *et al.* 2020): dissolved oxygen saturation and pH decreased by 10% but temperature remained unaffected. Ammonium and soluble reactive phosphorous increased 5.2 and 2.4 times respectively.

Sampling and sample processing

Biofilm

Before the start of the experiment, we deployed 50 artificial substrata (granite paving stones of 20 x 10 x 8 cm) along each reach (covering less than 0.5% of the streambed) to allow for biofilm colonization. According to Pereda *et al*. (2020), biofilm was formed by a similar amount of photoautotrophic and heterotrophic microorganisms, with a tendency to net autotrophy. In each sampling, we scrapped the whole surface of five randomly chosen paving stones and processed them to obtain ash-free dry mass (AFDM) per surface unit (g m^{-2}) (complete methodology in Pereda *et al*. (2020)).

Benthic organic matter and macroinvertebrates

We collected nine benthic Surber samples (surface of 0.09 m^2, mesh of 0.5 mm) randomly along each reach of the Apraitz Stream in each sampling. From each sample, the organic matter retained on an 8-mm sieve was gathered, leaves were separated from the rest of the coarse detritus and both categories were separately processed to obtain their AFDM in each sample (see Pereda *et al*. (2020)). Since consumption of wood and other recalcitrant materials by macroinvertebrates is negligible compared to leaves (Díez *et al.* 2002; Gulis *et al.* 2008) we only retained leaves (hereafter coarse detritus). Before getting the AFDM of coarse detritus, a subsample was kept for the assessment of Carbon (C) and Nitrogen (N) concentrations. Macroinvertebrates collected in a 0.5-mm sieve were preserved in 96% ethanol. In the laboratory they were sorted, identified to the lowest possible taxonomic level following Tachet *et al*., 2010 (mostly to genus-level except for some Diptera identified to subfamily level and Annelida to subclass level) and counted to obtain population densities. In addition, we measured the body length of up to 30 randomly selected individuals of each taxon in every sample (except for oligochaetes, planarians and leeches, which were not measured) with a binocular microscope (Leica M165FC, Wetzlar, Germany) equipped with a Leica DFC310FX camera using "Leica Application suite V4" software program. Total body length was considered as the distance from the anterior part of the head to the posterior part of the last abdominal segment excluding antennae and tails (Martínez *et al.* 2016). For gastropod molluscs, we measured the maximum length of the shell (Meyer 1989), and for crustaceans of the genus *Echinogammarus,* the dorsal length of the first abdominal segment was measured to posteriorly obtain body length (Flores *et al.* 2014). Individual body mass (BM) (mg dry mass) was derived using published length-mass relationships (Meyer 1989; Burgherr & Meyer 1997; Benke *et al.* 1999; Baumgärtner & Rothhaupt 2003; Stoffels *et al.* 2003; Larrañaga *et al.* 2009).

Fish

We conducted fish samplings along the 100-m long reaches by depletion electrofishing with a backpack-electrofishing unit with variable output current (MARTIN PESCADOR III, Alborlan S.L.). Stop-nets were set at the upstream and downstream ends of the reaches and up to three runs were made (Lobón-Cerviá 1991). All fish were anaesthetized with MS-222, identified, counted and weighed (to the nearest g). The study was approved by the Ethics Committee for Research Involving Animals at the University of the Basque Country (M20/2016/177). We converted wet mass into AFDM through conversion factors published in www.fishbase.se.

Quality of basal food resources

Six samples of biofilm were collected in each reach by scraping the entire surface of randomly chosen cobbles and collecting the slurry in filtered river water (0.7 µm pore size, Whatman GF/F). Six fine detritus samples were randomly collected per reach and period using a sediment corer (surface 81.7 cm^2). Biofilm and fine detritus samples were frozen (-20 °C) and freeze-dried (VirTis Benchtop 2K) (from 12 to 72 h depending on their water content). Each subsample of coarse detritus was oven dried (72 h, 70 °C). Freeze-dried and oven-dried material was ground (Vibration mill MM301, Fisher Bioblock Scientific) and weighed (approximately 10 mg for fine detritus and 2 mg for the other basal resources) into tin capsules (Lüdiwiss Sn 98, 5 x 8 mm) for C and N analysis. C and N analyses for biofilm and fine detritus were performed at the Helmholtz-Zentrum für Umweltforschung (Magdeburg, Germany) by a flash combustion on a Flash 2000 elemental analyser (Thermo Fisher Scientific, Bremen, Germany) and for coarse detritus at the Stable Isotope Facility of the University of California – Davis on a PDZ Europa ANCA-GSL elemental analyzer (Sercon Ltd., Cheshire, UK).

Data treatment

Invertebrate diversity

We assessed invertebrate taxa diversity through Hill numbers (i.e. number equivalent, ^{q}D (Jost 2006)) with the entropart package for R (Marcon & Hérault 2015). We used Hill numbers of order 0 (^{0}D, species richness, which is insensitive to the abundance of individuals of each taxon, highlighting the response of rare taxa), 1 (^{1}D, the exponential of Shannon's entropy, which weighs each taxon according to its log-transformed abundance), and 2 (^{2}D, inverse of Simpson concentration, which weighs each taxon according to its abundance, highlighting the response of dominant taxa) (Jost 2006). We computed alpha-diversity per sample in each period and reach for the three Hill number orders and beta-diversity among samples within each period and reach for orders 0 and 1 of diversity measures. Beta-diversity (D_{β}) for the diversity orders (q) 0 and 1 was transformed from beta entropy (H_{β}) as described in Marcon and Hérault (2015):

$$ {}^{q}D_{\beta} = e_q^{\frac{{}^{q}H_{\beta}}{1-(q-1)\,{}^{q}H\alpha}} \, . $$

Food webs, energy flux and dependency on basal resources

Following the number of replicates of Surber samples per period and reach we constructed 9 local food webs. Information about organic matter and invertebrates was obtained from the Surber samples. In the case of biofilm, we used the average biomass per paving-stone surface in each period and reach to estimate total biomass in each food web. Fine detritus was a scarce basal resource with a heterogeneous distribution along the reach and was not quantified during the sampling campaign, so it was equalled to the mean biofilm biomass values recorded. In the case of fish, assuming a homogeneous distribution of fish along the reach, we estimated the total biomass in each sample (Surber sampler area, 0.09m2). For every invertebrate taxon in each sample, we calculated mean BM from the individual masses measured and estimated the total biomass per node. We also estimated total metabolic rate (MR) for each invertebrate and fish nodes based on individual MR, calculated for each individual using an allometric equation derived from Gillooly *et al*. (2002) :

$$X = exp((a \cdot ln(BM) + x_0) + E/kT) ,$$

where X is the MR (in watts, W), a is the allometric exponent (0.71), BM is the body mass (g), E is the activation energy (0.63 eV), k is the Boltzmann's constant ($8.62 \cdot 10^{-5}$ eV K^{-1}), T is the temperature (K) and xo is a normalization factor (17.17 for invertebrates and 18.47 for fish). All these parameters were extracted from Brown *et al*. (2004). We gathered mean daily T of the 190 days before the sampling date in each reach.

To estimate energy fluxes between nodes of local food webs, we used an adapted food-web energetics approach (Barnes *et al.* 2018; Gauzens *et al.* 2019; Jochum *et al.* 2021) by means of the "fluxweb" package (Gauzens *et al.* 2019). This approach uses allometric scaling laws to quantify MRs (Brown *et al.* 2004). The model assumes a steady-state system, where the energetic losses of nodes in each food web, estimated by MR of consumer j (X_j) and predation on consumer j by higher trophic levels (k), need to be balanced by the energetic gains defined through resource consumption and assimilation (O'Neill 1969; Barnes *et al.* 2018). The flux of energy $F_{i,j}$ from resource i to consumer j was calculated as

$$\sum_i e_{ij} F_{ij} = X_j + \sum_k W_{jk} F_k$$

where e_{ij} is the efficiency in which consumer j assimilates the energy consumed from resource i. Energy fluxes to each consumer are defined as $F_{ij} = W_{ij} F_j$, where F_j is the sum of all the ingoing fluxes to consumer j and W_{ij} is the proportion of F_j obtained from resource/prey i, after scaling consumer preferences w_{ij} to the biomass (B) of the different resources/preys as:

$$W_{ij} = (w_{ij} B_i)/(\textstyle\sum_k w_{kj} B_k).$$

For that, an adjacency matrix with trophic links among all taxa present in our study and feeding preferences for each food resource was created based on the literature (Tachet *et al.* 2010; Gray *et al.* 2015) and our own gut content findings (I. de Guzman, unpublished data) (see in https://www.dropbox.com/s/owh3tnj7yulfaku/matrix1_Elgoibar_trophic_links_tachet.csv?dl=0). For carnivore taxa we assumed that preferences were equally distributed amongst prey species. For omnivore invertebrates and primary consumers w values were given following preferences in Tachet *et al*. (2010), where traits related to consumed food are quantified using affinity scores between 0 and 5. For omnivores, affinity scores related to predation were equally distributed amongst prey species. For cannibalistic species, we set the preference for cannibalism to 0.01 in the adjacency matrix to minimize the amount of energy a consumer could ingest from its own biomass pool. Assimilation efficiencies (Ɛ) for the consumption of food resources were calculated deriving a formula from Lang *et al*. (2017): $Ɛ=(e^{Ɛ'} \cdot e^{E(T-T0)/(kTT0)})/(1+(e^{Ɛ'} \cdot e^{E(T-T0)/(kTT0)}))$,

where Ɛ' is normalization constant for assimilation efficiency (-1.670 for detritivory, 0.179 for herbivory and 2.260 for carnivory), E is the activation energy (0.164 eV), k is the Boltzmann's constant and T is the temperature (K). Parameters were extracted from Lang *et al*. (2017).

We calculated whole-food web energy flux as the sum of energy fluxes within each local food web (each Surber sample). To calculate the consumption on biofilm, detritus and preys, we summed all the outgoing energy fluxes from each food resource. Thus, we quantified three consumption pathways: herbivory (consumption of biofilm), detritivory (consumption of coarse and fine detritus) and carnivory (consumption of animals) in the entire food web and within each trophic group (primary consumers, omnivores and carnivores) of each local food web. In addition, we calculated the dependency of each trophic group on basal food resources (biofilm and detritus) with the "NetIndices" package (Soetaert & Kones 2014).

Size spectra

We constructed size spectra for the entire community (including both invertebrate and fish assemblages), and for primary consumers, omnivores and carnivores, separately. We used BM of the measured (invertebrates) and weighed (fish) organisms. Since the log-transformed length values followed a normal distribution, we obtained BM of the remaining non-measured invertebrates by means of the "truncnorm" package (Mersmann *et al.* 2018), based on the mean, standard deviation, minimum and maximum values of each taxa in each sample. We used animals with BM higher than 0.1 mg to construct size spectra, since organisms with lower weights are undersampled as they are mostly washed through the 0.5 mm mesh aperture sieves (Gruenert *et al.* 2007). We divided the total range of BM ($\log_{10}$BM) values into 8 logarithmic bins of the same width and regressed density of organisms ($\log_{10}$Density) against the centre of the bin (White *et al.* 2008).

Statistical analyses

We conducted linear models with the factors period (Before and After), reach (Control and Impact) and their interaction as sources of variation (as well as the covariate BM for size spectra comparisons). This kind of BACI models do sometimes pose some challenges when graphically interpreting the results, as the stressor can dissipate instead of amplify initial differences between control and impact sites (see Fig. S1 in Supporting Information for an illustrative explanation). Coefficients for the interaction term point the direction of the effect of interest. Some variables were log-transformed to fulfil the requirements for linear models. We conducted all the analyses using R software, ver. 3.6.0. (R Core Team 2019).

Results

Stock and quality of basal resources

Coarse detritus stock tended to be higher in the impact reach ($F_{1,31}$ = 3.90, p = 0.057, Fig. S2, Table S1). Biofilm stock differed between periods ($F_{1,16}$ = 5.56, p = 0.031, Fig. S2), and suffered a marginally significant increase as a consequence of the addition of the effluent ($F_{1,16}$ = 3.84, p = 0.068, Fig. S2, Table S1). In addition, carbon-to-nitrogen ratios (C:N) (Table S2) significantly differed among basal food resources ($F_{2,75}$ = 240.8, $p < 0.001$), but the addition of the effluent affected none (Table S1).

Invertebrate and fish diversity and abundance

Alpha-diversity of macroinvertebrate taxa richness significantly differed between reaches (Fig. 2, Table 1) and decreased in the impact reach respecting the control reach with the addition of the effluent ($F_{1,30}$ = 16.07, $p < 0.001$, Fig. 2, Table 1). However, the effluent did not significantly affect Shannon and Simpson's diversity (Fig. 2, Table 1). Macroinvertebrate taxa richness at beta-diversity scale was initially higher in the control reach, but the difference disappeared with the addition of the effluent ($F_{1,124}$ = 27.89, $p < 0.001$, Fig. 2, Table 1). We observed a similar pattern for Shannon at beta-diversity scale: effluent addition led to less homogeneous communities ($F_{1,124}$ = 5.73, p = 0.018, Fig. 2, Table 1). Moreover, the effluent had a negative net impact on invertebrate density, as the initial differences found between the two experimental reaches decreased ($F_{1,30}$ = 4.76, p = 0.037, Fig. 2, Table 1). The responses in diversity of the entire invertebrate community reflected the responses observed in each trophic group (primary consumers, omnivores and carnivores), as diversity measures in all the groups responded similarly to the addition of the effluent. Regarding fish assemblages, four species were present during the study, and in both periods, more individuals and higher biomass were found in the impact reach, but overall, individuals at the control reach were bigger (Table S3).

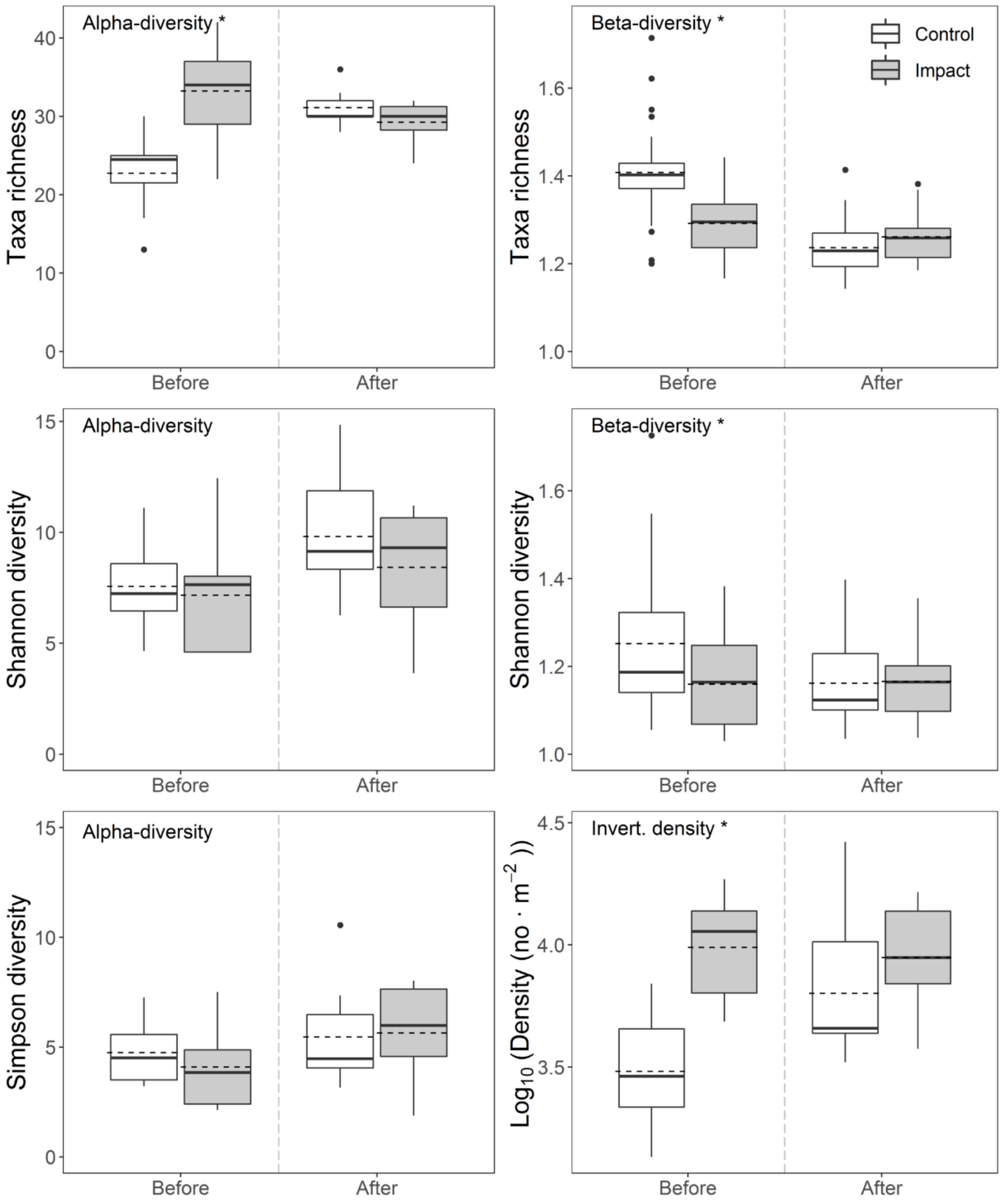


Fig. 2. Diversity and density of macroinvertebrate assemblage: alpha- and beta-diversity for taxa richness and for Shannon diversity, alpha-diversity for Simpson diversity and density. The box plots show the median, the interquartile range and the tails of the distribution. Dots represent outliers and dashed lines mean values.Asterisks indicate the effect of the effluent (Period:Reach interaction) was significant.

Table 1 Linear model results comparing invertebrate density and diversity, energy fluxes throughout food webs and dependencies of trophic groups on basal resources between control (C) and impact (I) reaches before (B) and after (A) the start of effluent addition to assess its effects (BA:CI). Values in bold indicate statistical significance ($p < 0.05$). Coefficients are shown for significant responses and consider B and C as reference in all cases. In dependencies on basal resources F and p values are identical within each trophic group because the sum of dependencies on biofilm and detritus equals 1.

		BA			CI			BA:CI		
		F	p	Coeff.	F	p	Coeff.	F	p	Coeff.
Invertebrate community descriptors										
Taxa richness	Alfa	1.6	0.216		7.83	**0.009**	10.47	16.07	**<0.001**	-12.33
	Beta	52.21	**<0.001**	-0.17	11.76	**0.001**	-0.12	27.89	**<0.001**	0.14
Shannon diversity	Alfa	4.31	**0.046**	2.25	1.06	0.311		0.32	0.574	
	Beta	3.35	0.069		4.81	**0.03**	-0.09	5.73	**0.018**	0.1
Simpson diversity	Alfa	2.76	0.107		0.12	0.734		0.38	0.542	
Invertebrate density ($\log_{10}$)		2.11	0.156		15.65	**<0.001**	0.51	0.51	**0.037**	-0.36
Energy fluxes										
Entire community		16.46	**<0.001**	0.35	71.6	**<0.001**	0.55	6.62	**0.015**	-0.26
Herbivory		8.66	**0.006**	0.04	11.62	**0.002**	0.06	7.65	**0.01**	0.53
Detritivory		8.83	**0.006**	0.47	50.86	**<0.001**	0.73	10.23	**0.003**	-0.45
Carnivory		6.2	**0.019**	0.26	8.44	**0.007**	0.28	1.45	0.238	
Dependencies on resources										
Primary consumers	Biofilm	0.32	0.578		0.38	0.543		2.41	0.131	
	Detritus	0.32	0.578		0.38	0.543		2.41	0.131	
Omnivores	Biofilm	0.45	0.507		0.23	0.633		3.2	0.084	
	Detritus	0.45	0.507		0.23	0.633		3.2	0.084	
Carnivores	Biofilm	0.32	0.579		0.61	0.44		7.66	**0.01**	0.13
	Detritus	0.32	0.579		0.61	0.44		7.66	**0.01**	-0.13

Energy fluxes and dependency on basal food resources

Mean BM, total biomass and MR of the three trophic groups did not change with the addition of the effluent (Fig. S3, Table S1). However, energy fluxes of the entire community differed between periods and reaches, and the addition of the effluent caused a negative net effect on them (Fig. 3, Table 2). Moreover, some fluxes were also sensitive to the effluent. Herbivory increased with the addition of the effluent (Fig. 3, Table 1), and in the after period differences in detritivory between reaches decreased (Fig. 3, Table 1). However, although carnivory significantly varied between periods and reaches, it was unaffected by the addition of the effluent (Fig. 3, Table 1). Omnivores were responsible for the increase in herbivory during the addition of the effluent, as energy fluxes significantly increased within this trophic group (Fig. S4, Table S1). On the other hand, the decreasing differences in detritivory between reaches after the addition of the effluent, were driven by the decrease in detritivory among primary consumers (Fig. S4, Table S1). Carnivory was unaffected in any trophic group with the addition of the effluent (Fig. S4, Table S1). Overall, all trophic groups depended on detritus more than on biofilm (Fig. 4). However, dependency of carnivores on biofilm significantly increased with the addition of the effluent, while dependency on detritus decreased (Fig. 4; Table 1), with no other group showing a variation on their dependency on biofilm or detritus.

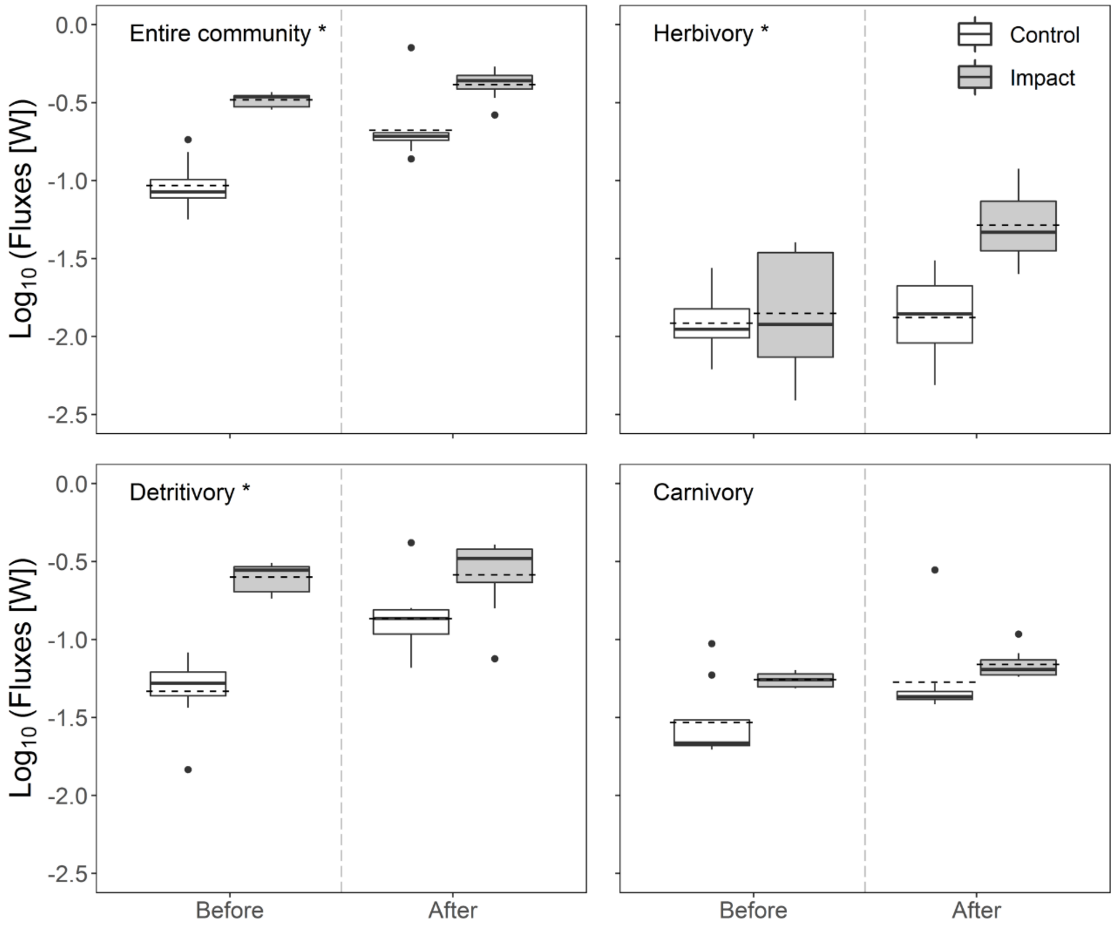


Fig. 3. Energy fluxes of stream food webs and functions: fluxes throughout the entire food web and herbivory, detritivory and carnivory related fluxes. The box plots show the median, the interquartile range and the tails of the distribution. Dots represent outliers and dashed lines mean values. Asterisks indicate the effect of the effluent (Period:Reach interaction) was significant.

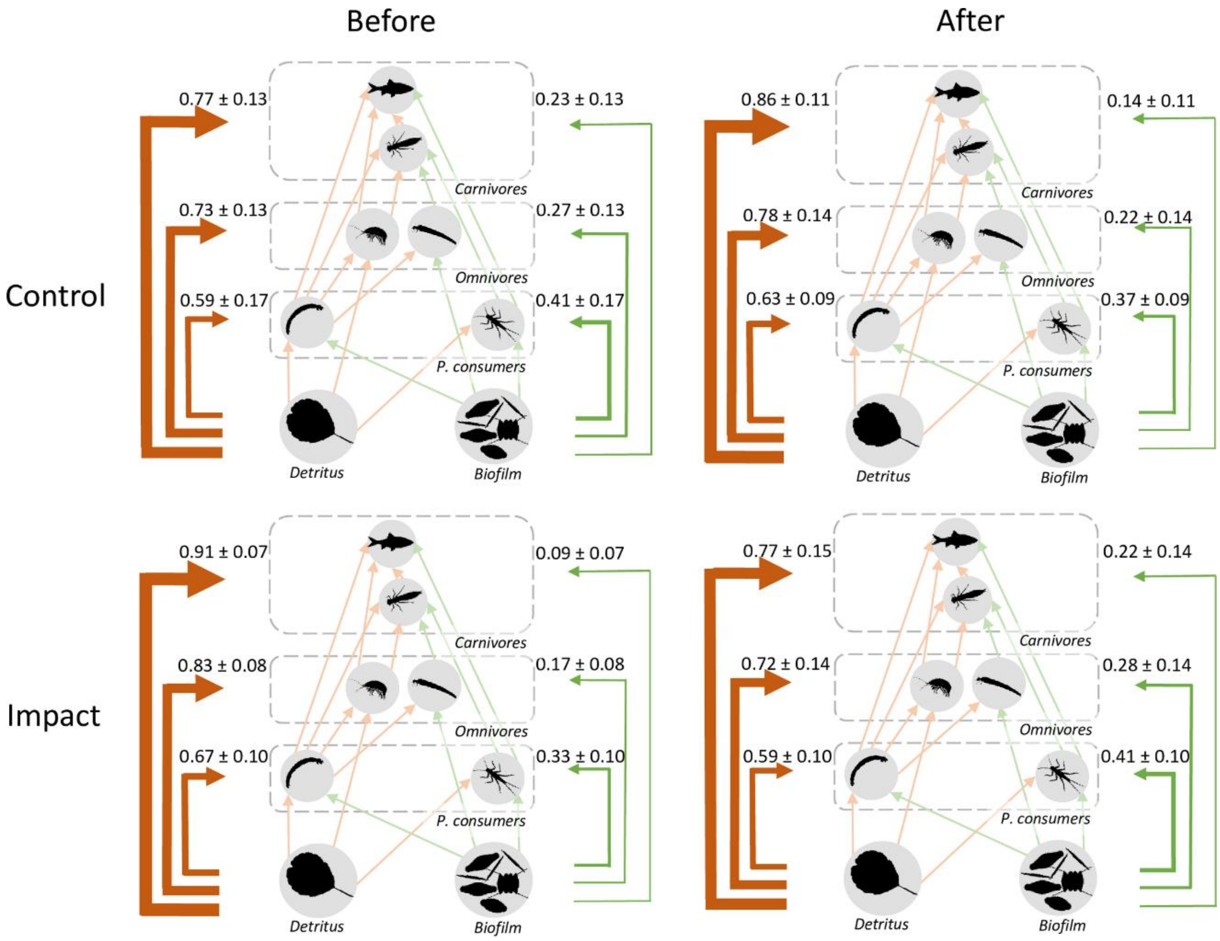


Fig. 4. Schematic figure of the dependency of each trophic group (primary consumers, omnivores and predators) on basal resources (detritus, orange arrows and biofilm, green arrows). Values indicate the average proportion and standard error.

Size spectra

The slope of the size spectrum of the entire community was unaffected by the addition of the effluent (Fig. S5, Table 2: Body mass:BA:CI, Fig. S6), as well as the slopes of the three trophic groups of consumers: primary consumers, omnivores and carnivores (Fig. S5, Table 2). Nevertheless, the addition of the effluent significantly reduced the intercept of the primary consumers (Table 2: BA:CI; coefficient AI: -0.636).

Table 2 Linear model results comparing size spectra between control (C) and impact (I) reaches before (B) and after (A) the start of effluent addition to assess its effects (BA:CI) and considering body mass. Values in bold indicate statistical significance ($p < 0.05$). Coefficients are shown for significant responses and consider B and C as reference in all cases.

	Entire community			Primary consumers			Omnivores			Carnivores		
	F	p	Coeff.	F	p	Coeff.	F	p	Coeff.	F	p	Coeff.
Body mass	304.20	**<0.001**	-0.95	164.43	**<0.001**	-0.78	245.34	**<0.001**	-0.79	66.70	**<0.001**	-0.28
BA	6.03	**0.022**	0.58	11.58	**0.002**	0.54	13.04	**0.001**	0.19	13.34	**0.001**	0.45
CI	0.02	0.888		0.13	0.722		21.15	**<0.001**	0.39	0.00	0.954	
Body mass:BA	0.16	0.695		5.99	**0.022**	0.13	0.86	0.364		0.00	0.968	
Body mass:CI	0.66	0.423		1.71	0.203		3.99	**0.057**	-0.27	0.87	0.359	
BA:CI	0.89	0.356		10.47	**0.004**	-0.64	1.54	0.227		0.91	0.350	
Body mass:BA:CI	0.04	0.844		2.07	0.163		0.13	0.720		0.10	0.757	

Discussion

WWTP effluents represent one of the main anthropogenic source of pollution to freshwater ecosystems (Rice & Westerhoff 2017). Their effects depend on effluent composition, dilution and the characteristics of the receiving ecosystem. Our whole-ecosystem manipulation experiment shows that even a well-treated and highly diluted (mean 3%, range 0.2-4%) effluent alters diversity and energy pathways on food webs.

The effluent promoted biofilm biomass

Although the effect of the effluent addition on biofilm abundance was marginally significant for the sampling campaigns in which food web energy fluxes and size spectra were quantified, it was highly significant when considering all the samples collected every second month during the experiment (see Pereda *et al.*, 2020). Biofilm growth is known to increase under enhanced nutrient concentration (Francoeur 2001; Ribot *et al.* 2015; Dodds & Smith 2016) and under sewage inputs (Pereda *et al.* 2019, 2021), where microbial communities composed by pollution-resistant taxa can form highly functional biofilm communities (Rosi *et al.* 2018).

Many studies have reported that increased concentrations of dissolved nutrients promote litter quality (Cross *et al.* 2003, 2006) and decomposition (Ferreira *et al.* 2014). Pereda *et al.* (2020) also observed an increase in detritus decomposition in the same experimental setup, although we did not observe changes in the quality of detritus in this study. Surprisingly, in presence of effluent, we did not observe alterations in the C:N ratio of biofilm either, which contrasted with other studies addressing effects of nutrient enrichment even downstream from effluent inputs (Scott *et al.* 2008; Lyon & Ziegler 2009).

Effects on diversity were scale-dependent

The effluent reduced invertebrate richness and density, but Shannon and Simpson at alpha-diversity scale were unaffected. Studies addressing WWTP effluent effects have reported increased invertebrate abundance and decreased diversity as a consequence of the loss of the most sensitive taxa and the stimulation of the resistant ones (Morrissey *et al.* 2013; Mor *et al.* 2019; Hamdhani *et al.* 2020; Weitere *et al.* 2021), indicating that these effluents act as environmental filters that select pollution tolerant taxa. The highly diluted effluent in our study did still negatively impact taxa richness at alpha-diversity scale. Contrarily, we observed that the effluent significantly increased beta-diversity, indicating increased spatial turnover across local communities. Mor *et al*. (2019) also reported an increase in beta-diversity between samples obtained in reaches downstream from WWTP effluents and attributed it to increases in local habitat heterogeneity, to opportunistic responses of biota to increased productivity or to basal environmental differences between sites. In our study, we could associate the increase in beta-diversity to the stronger role of stochastic assembly events caused by the increase in productivity (Chase 2010), which promotes the opportunistic responses of some species (Langenheder *et al.* 2012), and to the uneven elimination of some sensitive species to pollutants (Trubina & Vorobeichik 2012).

WWTP effluents have been reported to reduce the abundance and biomass of fish (Weitere *et al*. 2021) as well as fish diversity (Hamdhani *et al.* 2020). In contrast, in our study the effluent had no clear effects on fish diversity and densities, likely due to the high dilution rate. Overall, more and smaller individuals were found in the impact reach, but the effluent did not affect abundance nor BM.

Herbivory was stimulated, but total energy flux was reduced

We expected increased biofilm to promote the energy flux along the green food web, as it is of higher nutritional quality than detritus (Cross *et al.* 2005) and can sustain a high production of primary consumers (McCutchan & Lewis 2002). Several studies reported that nutrient addition increased secondary production as it increased the quality of basal resources (Cross *et al*., 2006; Demi *et al*., 2018; Eckert *et al*., 2020), and consumers preferred biofilm against detritus (Bumpers *et al*., 2017). We observed no increment in mean BM, total biomass or estimated MR for any trophic group. However, we observed a net decrease in the total energy flux of the food web and a change in the amount of energy provided to consumers through different pathways, with increased herbivory and reduced detritivory. A similar shift from brown to green food-web pathways was reported through the increase of herbivore abundance in a nutrient enrichment experiment where detritus was also the most abundant basal resource (Bumpers *et al*., 2017). In our experiment the energy fluxes towards omnivores and primary consumers shifted, although none of these groups increased in biomass or abundance. Omnivores, able to consume detritus, biofilm and preys, increased the consumption of high-quality biofilm, whereas primary consumers reduced the consumption of low-quality detritus. Carnivory did not increase, but carnivores became more dependent on biofilm, which shows that the effects of the effluent reached the top of the food web.

Community size spectra were unaffected

Stoichiometric ratios (C:nutrient) of basal food resources are generally higher than those of animals (Lemoine *et al.* 2014), which limits the growth of primary consumers (Evans-White & Halvorson 2017). Water nutrient enrichment can increase the quality (i.e. reduce C:nutrient ratio) of autotrophs and detritus (Xu & Hirata 2005; Evans-White & Halvorson 2017) narrowing the stoichiometric gap between basal resources and primary consumers and increasing the efficiency of trophic transfer (Mulder & Elser 2009). We did not see a shift in C:N ratio of basal resources, although we observed an increase of the relative abundance of biofilm over detritus, which enhanced the total availability of nutrients for consumers. Therefore, we expected a shallower slope in the BM-abundance size spectra (Xu *et al.* 2015) (or a steeper slope in biomass-abundance (Mulder & Elser 2009; Ott *et al.* 2014)), as nutrient subsidy favours organisms with higher per capita metabolic requirements (Cross *et al.* 2003) and increases the capacity of the system to support larger individuals (Juvigny-Khenafou *et al.* 2021).

However, the effluent did not change the slope of the size spectra. This finding could suggest that the nutrient enrichment was not high enough to trigger a change in energy transfer efficiency. Additionally, toxicity of the effluent might not have been strong enough to modify the slope either, as several studies observed size specific responses to pollution (Gergs *et al.* 2015; Taddei *et al.* 2021), small bodied organisms being more sensitive to toxicity (Kang *et al.* 2019) due to a higher specific metabolism and a greater surface to volume ratio (Wang & Zauke 2004). Thus, shallower slopes in size spectra might also be predicted with high toxicity. Nevertheless, the only sign of the effect of the toxicity of the effluent on size spectra was the lower intercept for primary consumers, which is in line of the subtle effects of treated and diluted effluent on ecophysiology of consumers (Solagaistua *et al.* 2018) and ecosystem functioning (Pereda *et al.* 2020).

Conclusion

Our whole-ecosystem manipulative experiment shows that even well treated and highly diluted WWTP effluents can modify the structure and functioning of stream communities. The reduction of alpha- and the increase of beta-diversity show that the effluent reduced the number of species, but increased the dissimilarity among the different patches of the riverbed. The overall size structure of the community did not change, energy fluxes through herbivory increased and those through detritivory decreased, but a negative effect on the total flux along the entire community was triggered. Our experiment underlines that current procedures to treat contaminated waters might not be enough to preserve natural properties of food webs and that further efforts might be needed when dealing with sensitive or highly valuable aquatic ecosystems.

Acknowledgements

This research was supported by the EU Seventh Framework Programme (GLOBAQUA; 603629-ENV-2013-6.2.1). Authors also acknowledge the financial support from the Basque Government (Consolidated Research Group IT951-16) and the pre-doctoral fellowship from the Basque Government (I. de Guzman). D. von Schiller is a Serra Húnter Fellow. J. M. Montoya is funded by the FRAGCLIM Consolidator Grant (number 726176) from the European Research Council under the EU Horizon 2020 Research and Innovation Program and by the "Laboratoires d'Excellences (LABEX)" TULIP (ANR-10-LABX-41). Authors are especially grateful to Libe Solagaistua, Olatz Pereda, Vicki Perez, Miren Atristain, Janire Diez, Madalen Goitia, Laura Sierra and Kelly Huamantumba for their assistance during field campaigns and laboratory analyses, and to the staff of the Apraitz WWTP for continuous support during the experiment. Mario Brauns and Ines Locker are also gratefully acknowledged for providing data on resource quality.

Conflict of interest

Authors declare that there is no conflict of interest regarding the material discussed in the manuscript.

Data availability statement

The original data that support the findings of this study will be openly available in Figshare and GitHub

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General Discussion

Overview of the main results

In the present dissertation, an observational field experiment and a whole-ecosystem manipulation experiment are combined to address the effects of two pervasive anthropogenic stressors for freshwater ecosystems on stream food webs (Table 1). The former experiment (Chapters 1 and 2), aimed at addressing the impacts of pollution and water diversion, on the structure and complexity of freshwater food webs. For this experiment, we selected four rivers that differed in their ecological status and water quality, which had a similar water diversion scheme, consisting of a low weir (3-6.5 m high) and a canal diverting part of the river flow to hydropower plants (Fig. 1). Food webs upstream and downstream the weirs were compared in a gradient of pollution which ranged from low to moderate. Each stressor induced different changes at the base of the food web: pollution increased the availability of biofilm and water diversion reduced the stock of coarse detritus downstream from dams. When assessing the contribution of these resources into primary consumers' diet (Chapter 1) or when quantifying the energy transferred through each food web pathway (Chapter 2), the brown pathway showed a consistent response, decreasing with the reduction of detritus stock. However, contrary to what we expected, the relevance of the green food web did not increase along the gradient of pollution. Invertebrate community did not show large variations regarding density and diversity with increasing pollution, although communities became more homogeneous (Chapter 2). Still, trophic diversity of the common taxa within rivers increased, indicating that they became more generalists (Chapter 1). The largest alterations were observed in presence of both stressors, which indicated that pollution exacerbated the effects of water diversion.

Fig. 1. Dams and studied reaches of the selected rivers ordered from lower to higher levels of pollution. Photographs provided by Vicki Perez.

Bearing this in mind, a whole-ecosystem manipulation experiment (Chapters 3 and 4) was conducted to address the effects of point source pollution on food web properties. Following a BACI experimental design, which allows controlling spatial and temporal variability, alterations induced by a properly treated effluent were detected (Fig. 2).

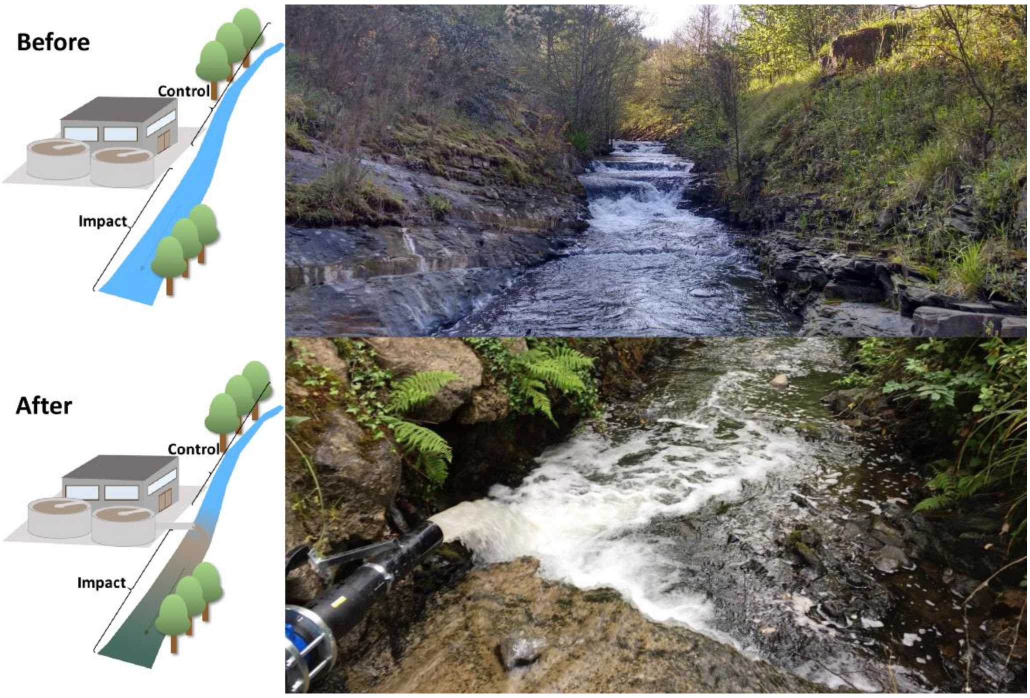

Fig. 2. Conceptual figure of the BACI (Before-After-Control-Impact) experimental design. The Control reach is upstream from the effluent pouring location. The Impact reach receives treated effluent during the After period. Photographs show the Control Reach and the effluent pouring pipe during a release ocassion. Photographs are provided by Miren Barrado and Arturo Elosegi.

Although being highly diluted (Pereda *et al*., 2020) (Fig. 3) and showing low toxicity for microbial performance and detritivore growth (Solagaistua *et al*., 2018), changes at the base of the food web were evident with the addition of the effluent and promoted the fluxes towards the green pathway (Chapters 3 and 4). When considering a long term time span, no meaningful changes were observed neither in diversity nor in abundance, although a drop of sensitive taxa and an increment of the resistant ones were observed (González *et al*., *In prep*). However, considering only the time span with the highest effluent contribution (Chapter 4), a decrease in invertebrate density and taxa richness was observed, which lead to increasing heterogeneity in the altered reach. These changes in the community help explaining the reduced energy fluxes in the entire food web through the brown pathway despite the promotion through the green pathway. Differing from Chapter 1, in Chapter 3 the entire community was considered in order to assess the changes in food web complexity. Trophic diversity of the entire community suffered an overall reduction with the effluent, which went against the expectations made beforehand. By narrowing down again the time span to the highest effluent contribution period, we observe that the unexpected decrease of trophic diversity can be linked to the decreased density and richness, which also leads to the increase in community redundancy. Still, additional analysis of the remaining seasons would be worth doing, to clearly see if the patterns observed in trophic structure go in line with community descriptors, or additional factors could be influencing the observed patterns.

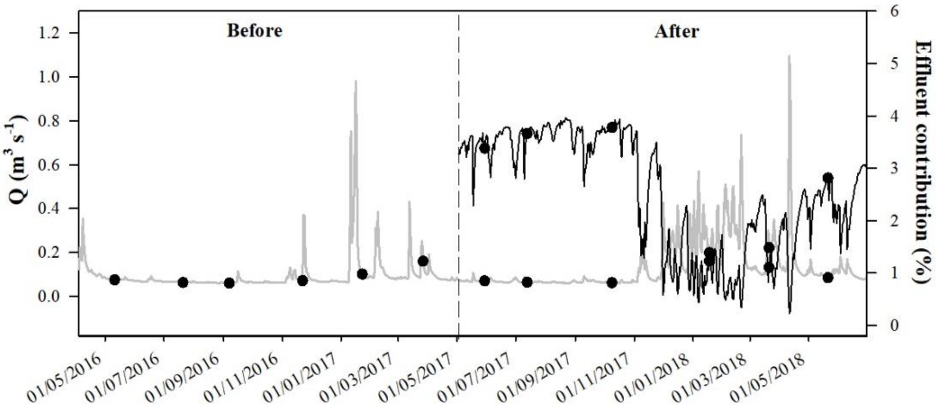

Fig. 3. Daily mean stream discharge (Q) in the Control reach (m3 s-1) (grey series, left axis) and effluent contribution to the Impact reach during the After period (%) (black series, right axis). The dashed line marks the beginning of the effluent addition. From Pereda *et al*., 2020, Figure S1.

Table 1. Main results obtained in this dissertation: effects of pollution, water diversion and their interaction on the response patterns of each variable. Arrows indicate the direction of the significant responses, an increase (pointing upwards) or a decrease (pointing downwards) and a lack of effect is indicated by the "≈" symbol. Green colour indicates the confirmation of the predicted effect, red indicates the predictions were not observed, and black is used when no predictions were made.

		Observational experiment						Manipulative experiment	
	Stressor	Pollution		Diversion		Pollution and diversion		Pollution	
	Chapter	Chapter 1	Chapter 2	Chapter 1	Chapter 2	Chapter 1	Chapter 2	Chapter 3	Chapter 4
Availability of basal food resources	Biofilm biomass	↑		≈		≈		↑	
	Detritus stock	≈		↓		≈		≈	
Energy pathways	Green pathway	↓	≈	↓	≈	↑	↓	↑	↑
	Brown pathway	≈	≈	↓	↓	≈	↓	↓	↓
	Total energy fluxes		≈		↓		↓		↓
Abundance	Invertebrate densiy		≈		↓		↓		↓
	Size-spectra		≈		≈		≈		≈
Diversity	Taxa richness (α)		≈		≈		↓		↓
	Taxa richness (β)		↓		↑		≈		↑
	Shannon diversity (α)		≈		≈		↓		≈
	Shannon diversity (β)		↓		≈		≈		↑
Food web complexity	Maximum FCL	↑		≈		≈		≈	
	Trophic diversity	↑		↑		↑		↓	
	Trophic redundancy	↓		↑		↓		↑	

Same stressor and contrasting responses

Pollution, one of the most pervasive stressors for freshwater ecosystems (Malmqvist and Rundle, 2002; Reid *et al*., 2019), is the common thread of this dissertation. As time went on and the results of the two experiments came into view, especially at the moment of joining all the chapters together, contrasting responses regarding this stressor emerged between the observational and the manipulative experiment. Changes at the base were consistent in both experiments, however, indeterminacies in further responses might have been given by differences in the interaction strengths between different components of the food webs.

Biofilm was a community compartment showing the same response in both experiments. Stream biofilm, processes and transforms nutrients and organic matter from the water column and the benthos with a combination of metabolic processes (Battin *et al*., 2016). With nutrient enrichment in freshwaters, one of the main constraint for the biofilm is relaxed (Marcarelli *et al*., 2009), which leads to a promotion in primary production (Keck and Lepori, 2012). However, a non-linear effect of nutrient pollution on biofilm production is frequently described, where stimulating effects are observed at moderate levels of nutrients (e.g. Ardón *et al*., 2021; Pereda *et al*., 2020; Ribot *et al*., 2015), but inhibitory responses are common at higher concentrations (Pereda *et al*., 2019). When assessing the effects of pollution in the present dissertation, an increase in biofilm accrual was observed in both experiments, indicating that the subsidy effects overrode detrimental effects on biofilm.

Since these changes in biofilm biomass increase initial energy flow at the base of the food web (Canning and Death, 2021) and can be propagated towards higher trophic levels (Ardón *et al*., 2021), we expected **green food web** to gain importance with increasing pollution, response that we observed in the manipulative experiment (Chapters 3 and 4), but not in the observational one (Chapters 1 and 2). In the later mentioned experiment, autochthonous resources seemed to be the main contributors to the diets of the selected primary consumers (Chapter 1), although entire communities showed a mayor dependency towards detritus (Chapter 2). The weak response of herbivory to pollution within the observational study (Chapter 2) can be linked to the non-significant variation of abundance. However, the non-significant positive patterns between pollution and herbivory or pollution and abundance in the control reaches suggests that the opposite trends observed in the reaches impacted by water diversion might be partially concealing the expected responses to pollution.

The pattern observed between pollution and taxa richness was similar to the just mentioned tendencies for abundance and herbivory (Chapter 2). This tendency contrast with the frequently described global trend for **local diversity** (i.e. species richness and diversity at α scale), which is reported to decrease as consequence of increasing habitat degradations (Loreau, 2010). A reduction in local diversity was however observed when assessing the interactive effects of pollution and water diversion (Chapter 2), and also when studying the alterations of the poured effluent (Chapter 4). These contrasting patterns in local diversity, reflect the current debate regarding the effects of anthropogenic stressors on local diversity (Norberg *et al*., 2022), which highlights the importance of conducting studies on the effects of global change components also at larger diversity scales, especially in ecological communities facing large environmental disturbances (Mori *et al*., 2018). Regarding **regional diversity** (i.e. diversity at γ- and β- scales), although it is frequently assumed that communities facing the same impact will tend to homogenization (i.e. getting a reduction in β-diversity) (e.g. Passy and Blanchet, 2007; McGoff *et al*., 2013), the opposite pattern has also been reported with anthropogenic impacts (Hawkins *et* *al*., 2015; Socolar *et al*., 2016). In our study, we observed a homogenization of the community with pollution (Chapter 2), whereas, on the contrary, the effluent addition increased it (Chapter 4). Thus, these results also emphasize that the effect of the same anthropogenic disturbance on assemblage composition can be variable, and depend on many factors (Hawkins *et al*., 2015), for instance, the magnitude of the impact or its characteristics (e.g. the concentration of the different pollutants and the synergies between them), the uniformity of environmental alterations (e.g. pollution might not be equally distributed in the entire impact reach in the manipulative experiment), and the initial ecological condition. In line with this, communities in the observational experiment might have already been in a stable state due to the long term disturbance, whereas the community of the manipulative experiment could have been in a dynamic transient state with the community accommodating to the recently induced stress. As perturbations are not equally distributed over species, with abundant species governing the short term responses and rare species gaining importance in long-term recoveries (Arnoldi *et al*., 2018), differences on direction or intensity of the response in the short- and the long-term can be behind the unequal response to pollution in our experiments.

These discrepancies on the response of diversity can also be linked to inconsistencies regarding **trophic structure** changes (i.e. trophic diversity and redundancy). However, we need to bear in mind that different approaches were used in the observational experiment (Chapter 1), where a representative subset of the community was used, and the manipulative study (Chapter 3), which showed the changes of the entire community, making the straightforward comparison between the two chapters challenging. Still, the most unexpected results regarding these variables were the ones reported in the manipulative study in which trophic diversity was reduced with pollution. Contrarily, as moderate nutrient pollution has been associated to a larger isotopic variability (Parreira de Castro *et al*., 2016; García *et al*., 2017) due to the increase of the isotopic diversity of basal resources, we expected an increase in trophic diversity. Although previous studies with the same effluent have reported low toxicity for microbial performance and detritivore growth (Solagaistua *et al*., 2018), the diversity decline during the highest effluent contribution period observed in Chapter 4 could be behind these changes.

Traditionally, a larger productivity in ecosystems has been linked to **longer food chains** (Pimm, 1982), and as expected, an increase in maximum food chain length (FCL) was observed with increasing pollution in Chapter 1. Nevertheless, a general increase in secondary production was not observed for the same experimental setup in Chapter 2 (an overall absence of responses in abundances and biomass was reported, despite the increment in certain trophic groups), and thus, a higher community individual turnover rate in the most polluted streams could have occurred (Woodcock and Huryn, 2007). This higher turnover could have enabled the observed increase in FCL along the pollution gradient. However, in Chapter 3 FCL did not follow the same pattern, as it was unaltered. Other factors apart from productivity can also influence FCL, such as disturbances and ecosystem size (Post, 2002). In small ecosystems where the spatial compression might be strong and productivity is not a limiting factor, ecosystem size could constrain FCL (Ward and McCann, 2017). In our experiment on the one hand, the tertiary treated effluent showed a low toxicity for microbes and detritivores (Solagaistua *et al*., 2018), was highly diluted in the receiving stream (Pereda *et al*., 2020) and thus, produced a low disturbance in the impacted reach. On the other hand, the availability of basal resources along the study site was high. Therefore, ecosystem size might have been responsible of shaping FCL in Chapter 3 rather than factors such as ecosystem productivity or disturbance, as fish in the impact reach could easily get into the larger Deba River.

Nutrient inputs are reported to narrow the stoichiometric gap between consumers and their resources (Mulder and Elser, 2009a), affecting in turn trophic transfer efficiency (Mulder and Elser, 2009a; Ott *et al*., 2014; Xu *et al*., 2015). These alterations in efficiencies can be observed through changes in the slope of the abundance-mass scaling relationship on a log scale of the community (i.e. **community size-spectra**) (Trebilco *et al*., 2013), with shallower slopes indicating a more efficient energy transference towards higher trophic levels, and thus supporting higher abundances of large species (Woodward *et al*., 2005a). Toxic compounds can also affect the size spectra not only through changes in the abundances of pollution-tolerant and sensitive taxa (Peralta-Maraver *et al*., 2019) but also due to size specific responses to pollutants (Gergs *et al*., 2015; Taddei *et al*., 2021), as small bodied organisms are more sensitive to toxicity (Kang *et al*., 2019) due to a higher specific metabolism and a greater surface to volume ratio (Wang and Zauke, 2004). However, no changes were observed in the size spectra in these experiments. In part, this lack of response could be related to the high dispersion of the largest organisms (i.e. fish) that impeded significant responses to appear. The high mobility of fish and, thus, the difficulty to estimate their mean density in a steady state can be behind this variability.

Methodological considerations

The present dissertation dealt with a wide range of data analyses together with standardized and replicable sampling methodologies. However, we are aware there are some limitations regarding some of the methodologies followed.

What should we consider an available resource for communities?

In small forested streams detritus is the main food resource, mainly in the form of leaf litter, (Vannote *et al*., 1980; Zhang *et al*., 2019) because of the frequent limitation of primary production by canopy cover (Bernhardt *et al*., 2018) and nutrients (Tank and Dodds, 2003; Elser *et al*., 2007). Although, this detrital organic matter colonized by microbes, constitutes the base of what is coined the brown food web (Marks, 2019), algae also play a key role in food webs due to their higher quality (Brett *et al*., 2017). Nonetheless, depending on the stream characteristics, light and nutrient availability, other basal food resources such as macrophytes, bryophytes or filamentous green algae can also contribute into stream food webs (Elosegi and Sabater, 2009). The availability of these resources however, might vary not only along the river continuum but also within a certain reach (Elosegi and Sabater, 2009), showing a different availability for the organisms present in the different patches, especially for the more sessile ones. This availability of basal food resources has turned out to be of great importance in the current dissertation, as different decisions had to be made regarding the available resources to carry out the analyses of interest.

When working with stable isotope data, the contribution of resources to primary consumers was estimated through Mixing Models (Stock and Semmens, 2017), which generally estimate proportions of assimilated resources with substantial uncertainty distributions (Phillips *et al*., 2014). For these analyses it is critical to sample all the available resources since the exclusion of a food source will bias the proportions for the other sources, producing inaccurate results when all the resources are not provided (Phillips, 2012; Phillips *et al*., 2014). Resource grouping might also influence the results, and thus, groupings making intuitive biological sense are recommended (Phillips *et al*., 2014). Following these advises, we sampled every available basal food resource in each reach regardless of their abundance, and decided to group them a posteriori as coarse detritus, fine detritus and autochthonous food resources. These decisions enabled us comparing the changes in contributions between the three main groups, as they were always available during the samplings and allowed us running statistical comparisons that could not be included beforehand due to the limitations of the Mixing Models. These decisions however, have probably influenced the obtained results, since some of the resources sampled in some reaches might not be as readily available as others (macrophytes or bryophytes, for instance), but were equally introduced into the models as suggested. One possible alternative could be to sample resources at patch scale instead of at reach scale to build mixing models considering the resources and consumers of each sample and combine the estimates of all the replicates per reach later on.

Constrains of working with individual body-size related data

The current dissertation encompasses a great effort in terms of collecting individual body size information, as assessing the effects of the stressors on the energy transfer of food webs was one of the main objectives. I has been widely observed that changes in nutrient availability alter the trophic transfer efficiency between resources and their consumers due to modifications in the stoichiometric gap (Mulder and Elser, 2009b; Ott *et al*., 2014; Xu *et al*., 2015). Therefore, we expected these changes to be also evident in both experiments of this thesis. Surprisingly we did not observe any alterations regarding the trophic transfer efficiency, although in some occasions changes in the intercept (i.e. mass corrected abundance) were described. Although the stressors studied might not be strong enough to create changes in energy transfer efficiency, we might also be facing a methodological constrain. As home range scales with body mass (Minns, 1995), the difficulty to detect a response might be inherently linked to the mobility of the largest organisms (Minns, 1995) and the consequent temporal fluctuation of the abundances and body masses in our reaches. The sampling of the largest consumers, fish, can easily be biased from the average abundance or body mass distribution for the period of interest. This might the reason behind the large dispersion of the largest body mass categories. For macroinvertebrates the larger replication of invertebrate samplings (nine replicates per campaign and reach) and the smaller home range have proven to be good ways to achieve linearity (see Fig. 6b in Chapter 2, Martínez *et al*., 2016). Additional samplings in time for the fish assemblage would probably increase the accuracy of mean abundance and body mass distribution estimates, and thus, facilitate the detection of significant responses to stressors.

The accuracy of abundance and body mass distribution average values also influences the calculations of the energy fluxes based on the biomass of each food web node. Biomasses were calculated from mean body mass and abundance of each taxa for invertebrates, and summing the measured body masses of each species for fish. The same problem that we acknowledge for size spectra (only one estimate for species with large home ranges) applies for energy fluxes. Again, additional fish samplings, would have allowed to have a better estimate for the fish assemblage.

Implications for management

In order to preserve the good status of freshwater bodies, several environmental laws such as the European Water Framework Directive (2000/60/EC) or the European Urban Wastewater Directive (91/271/EEC) were implemented. Consequently, in the last decades many WWTP have been set into operation or upgraded (Serrano, 2007; Langergraber *et al*., 2018; Mas-Ponce *et al*., 2021) with the objective of dealing with urban and industrial polluting effluents. WWTPs need to release treated effluents that are within the emission limit values for specific contaminants regulated under specific legislations (e.g. Water Framework Directive (2000/60/EC), Nitrates Directive (91/676/EEC), and Dangerous Substance Directive (67/548/EEC)) before releasing into the environment. However, although complying with these laws greatly reduce the amount of contaminants reaching aquatic ecosystems (Vaughan and Ormerod, 2012; Brion *et al*., 2015), treated sewage still consists of a complex mixture of pollutants, nutrients, and pathogens (Pascual-Benito *et al*., 2020). Additionally, the amount or the impact of pollutants in waterbodies might also depend on external factors, such as the reduced dilution capacity of freshwaters due to low discharges (Rice and Westerhoff, 2017) or the intermittent release of untreated sewage due to rainy episodes (Corada-Fernández *et al*., 2017). These circumstances together with the effects described in our manipulative study with highly diluted WWTP effluents, underline that current procedures to treat contaminated waters might not suffice when natural properties of food webs are intended to preserve. Our study highlights that further efforts in wastewater treatments might be needed especially when dealing with sensitive or highly valuable aquatic ecosystems. We have seen that stimulation of biofilm growth is responsible for the changes in the food web, and thus, tertiary treatments that further reduce the loads of nutrients can be part of the solution. Water ecosystems have the capacity to assimilate pollutants and consume nutrients, increasing water quality (Zubaidah *et al*., 2019). In lotic ecosystems this ability can constraint the negative effect of the effluent. Nevertheless, effluents were not released within the reaches of our observational studies, and yet, nutrient loads were different, with obvious effects of upstream pollution releases (either point source or diffuse) on the chemistry of the water, irrespective of any depuration that the ecosystem was exerting. The implementation of stronger restrictions from the already existent directives would additionally improve the quality of the poured effluent. Implementations of more WWTPs could help alleviating the load in the existing plants and allow for longer sewage incubation periods for driving a greater reduction of contaminants. Additionally, separating wastewaters depending on their origins (i.e. pluvial vs. faecal) could help adequately processing each type of effluents depending on their compositional characteristics. This would be truly beneficial, especially in rainfall events where black waters could keep receiving a suitable treatment despite the increased loads of pluvial waters. Diffuse pollution is also of great concern (Campbell *et al*., 2005). Nature based solutions such as riparian fencing, buffer strips or constructed wetlands have resulted to be good tools to reduce the impact of this type of pollution (Williams *et al*., 2020). The costs and efforts involved in implementing these measures are high; however, the advantages for the good preservation of freshwater habitats are worthwhile.

On the other hand, water diversion is a necessary practice to fulfil the growing demands of water and energy supplies. The increasing human populations is leading to the creation of more barriers with this purposes (Belletti *et al*., 2020), resulting in an increasing number of obstacles and alterations for lotic ecosystems. The current work shows that beyond the frequently described impacts on the physical structure and mobility patterns of river biota, strong modifications at the base of the food web can occur, which can also be transferred across the food web. These structures, however, can loss their function, become obsolete or non-viable from an economic perspective (Pohl, 2002). Removing these barriers could help restoring physical and ecological attributes and functions of these ecosystems. Although the increased trend in dam removal during the last decades is noticeable (Foley *et al*., 2017), larger efforts should be done to return the connectivity of rivers when these structures are no longer in use.

Further considerations

The present dissertation combined observational and manipulative experiments, and although it was adequately developed, it was necessarily limited in extent: this work addresses the effects of two anthropogenic stressors in a small geographic area, for the experiment consisting of several sampling locations a single sampling in time was performed, whereas for the manipulative experiment no replications in space were made although an exhaustive sampling along time was conducted. These methodological constrains, may affect the conclusions especially when trying to speculate about the global magnitude of these stressors. Therefore, some further considerations are addressed here before going beyond the obtained results and drawing general conclusions.

Replication of the experiments

The four sampling locations selected for the observational experiment allowed us creating a pollution gradient to assess the interactive effects of pollution and water diversion. This amount of spatial replicates however, are not enough for a reliable discrimination between linear and non-linear curve fitting (Jenkins and Quintana-Ascencio, 2020), which is a limitation for the kind of statistical analyses we can implement and, thus, for the predictive power we can achieve. However, finding study sites with a similar diversion scheme which only differed in the pollution level was impossible in our region. Additionally, spreading into other climatic regions apart from a significant increase of the sampling effort, would have probably included additional confounding factors into the study, complicating the evaluation of the impacts of interest and the interpretation of the results.

In addition, there are many studies assessing the strong effects of poorly treated and highly concentrated effluents in freshwater communities, who have included several study sites to accurately describe the impacts (e.g. Morrissey *et al*., 2013; Mor *et al*., 2019). However, the effects that well-treated and highly diluted effluents might produce have not been so well studied due to the co-occurring stressors these ecosystems frequently face or due to the complications of isolating the impacts of interest from confounding factors related to spatiotemporal variability. In this dissertation a case study was conducted in order address this knowledge gap. In the trade-off between selecting multiple sampling locations or conducting what to our knowledge was an unprecedented manipulative study, we were inclined towards the latter option, since even low pressures can produce clear changes on ecosystems (Hillebrand *et al*., 2020), but they can go unnoticed without an experimental design that can adequately isolate the effects from spatiotemporal variations. Now that we have successfully detected the response to this kind of subtle effects, a call for further studies can be done to cover the expected variability regarding other geographical areas.

Would the effects of these stressors be similar in other geographical areas?

The studies presented here were carried out in the temperate region of the northern Iberian Peninsula, a mountainous and industrial area with wet climate due to the frequent precipitations all over the year. The characteristics of this region have certainly influenced the responses observed in each experiment. When dealing with multiple stressors (Chapters 1 and 2), we highlighted the importance of ranking the stressors depending on their ecological relevance apart from describing the interactive effects they might show. In our observational study, pollution seemed to modulate the effects of water diversion. However, in the current global change scenario, droughts are becoming more frequent and lasting, which may intensify the impacts produced by water abstraction in rivers (Stevenson and Sabater, 2010). Strong droughts may alter the underlying structure and functioning of food webs, by triggering substantial losses of species and their interactions, and affect fluxes along food webs (Ledger *et al*., 2013). Nonetheless, long exposures to one stressor might cause adaptations of the community replacing sensitive with tolerant species (Petrovic *et al*., 2011), as is the case of communities in semiarid regions, who show an adaptation of communities to droughts (Bonada *et al*., 2007; Lawrence *et al*., 2010). Yet, consequences could also be more detrimental due to the vulnerability of communities already facing other stressors (Filipe *et al*., 2012). Additionally, changes in the flow regime can produce differences in the exposure of communities to pollutants, with low flows increasing the contact between toxicants and organisms (Petrovic *et al*., 2011). In line with this, in our manipulative experiment (Chapters 3 and 4), we described a low contribution of the effluent to the general discharge of the studied stream (Pereda *et al*., 2020), and thus, our results are not directly transferrable to other scenarios with much larger contribution of effluents, i.e. less diluted effluents. Nevertheless, although being an effluent that received tertiary treatments and the high dilutions in the stream, changes in food webs were obvious. Pouring an effluent of the same characteristics into a stream with lower flows, would have certainly increased the impact, as lower flows reduce the dilution capacity of streams and increase the concentrations of pollutants (Rice and Westerhoff, 2017).

Would impacts decrease downstream?

Both field experiments described in this dissertation addressed the effects of two stressors in 100-m long stream reaches immediately below the induced impact. However, these effects might have been traceable downstream from the studied reaches. Works assessing the effects of dams frequently conduct longitudinal samplings (e.g. Mor *et al*., 2018; Dolédec *et al*., 2021) to properly describe the magnitude of the produced impacts along the river, as the inputs of ground water and tributaries might reduce the impacts downstream. Still, some diversion schemes often impede the incorporation of any tributary into the main channel (Izagirre *et al*., 2013), entailing a great flow reduction all along the diversion canals. Thus, as the flow reestablishment greatly differs among streams, the repercussion on communities may also vary. Longitudinal approaches have also been used to assess effects of pollution in these ecosystems (e.g. Weitere *et al*., 2021; Freitas *et al*., 2022). These water bodies have a self-depuration capacity, which depends on the characteristics of the river such as on the flow velocity, water discharge volume and initial pollutant content, as well as on biotic factors (Zubaidah *et al*., 2019). Thus, assessing how communities change along the recovered reach could also be interesting from a management perspective, but that objective falls out of the scope of this project.

Do impacts depend on the seasonality?

When we addressed the impacts of pollution and water diversion (Chapters 1 and 2), we conducted a single sampling campaign in each stream. The samplings were carried out in a period when the largest flow differences between upstream and downstream reaches from diversion dams occur because precipitation rates are lower, but diversion canals are still active. However, freshwaters are dynamic ecosystems that undergo seasonal changes in hydrological and biotic characteristics (Woodward and Hildrew, 2002; Woodward *et al*., 2005b; Power *et al*., 2008). Therefore, a single sampling in time might represent a somehow biased picture of the food web dynamics. Additionally, it is well known that food web ecology faces a great challenge regarding the quantity and quality of required data to document species richness and the interaction strength among them (Rooney *et al*., 2008). Although initially ideal, including spatial and temporal variation to get a more thorough understanding of food webs is an additional difficulty this field of knowledge confronts due to the excessive effort involved (Rooney *et al*., 2008). In our observational experiment where the main objective was to assess the interactive effects of pollution and water diversion on food webs, we necessarily had to focus the sampling campaign in the most divergent period. Adding subsequent samplings to assess the seasonal variation in food webs during the condensed time span of the thesis, would necessarily require limiting the effort on other interesting aspects of the project as a simplification of, or even not considering, the pollution gradient or only considering the effects of pollution. A similar constrain was faced in Chapter 4. Although a highly-resolved community structure has been obtained for the manipulative experiment during the bimonthly conducted samplings (González *et al*., *In prep.*), including individual measurements for the accurate estimation of energy fluxes for each sampling would have exceeded the efforts that could be made in this project. Thus, here again, in a trade-off to achieve the most accurate information for the targeted variables, a single period was chosen, when the effluent had its highest contribution.

Future directions

- Further research on food webs facing a wide range of multiple stressors through global collaborative projects would help understand the implications of combining anthropic environmental drivers on biodiversity and ecosystem functioning. These projects would describe the isolated and interactive contribution of the stressors and help develop measures to mitigate global change.
- Assessing the link between the effects of multiple stressors and temporal dynamics (seasonality, life cycles, extreme events...) in food webs would enable identifying the key moments in which food webs are more or less resistant to change. This will also allow discerning between the direct effects of the stressors on food webs and the secondary changes due to species interactions.
- Comparing the effects of one stressor continuously affecting a food web (press perturbation) with the same stressor showing an intermittent impact (pulse perturbation) through long term studies would help understand how the effects are propagated along food webs. These studies will give insights on the stages towards recovery or stabilization of natural food web properties.
- Analyses of long term biomonitoring data with food web approaches (size spectra, energy fluxes...) will also help disentangling the implications of each stressor within the global environmental change and allow predicting future scenarios. This scenarios would allow anticipating to the change and applying mitigating measures.

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General Conclusions

Combined effects of pollution and water diversion on freshwater food webs:

1. Our observational experiment showed complex responses of food webs to pollution, water diversion and their interaction, with pollution enhancing most of the responses to water diversion. (*Chapters 1 and 2*)
2. Pollution increased biofilm biomass, and although it decreased the importance of the green pathway, observed increases in primary productivity increased food web complexity by increasing trophic diversity and enabling longer food chains.
3. Water diversion reduced the stock of organic matter, producing a decrease in the energy transfer through the brown pathway, a decrease in invertebrate density, and an increase in both trophic diversity and community taxonomic heterogeneity.
4. The interaction of pollution and water diversion produced even larger effects on food webs, despite the combination of both stressors did not further affect the base of the food web.

Main effects of pollution in the manipulative experiment:

1. Our field manipulative experiment emphasized that even tertiary treated and highly diluted effluents can generate complex responses on food web structure and functioning, which could have remained unnoticed under less sensitive experimental approaches. (*Chapters 3 and 4*)
2. Effluent addition promoted biofilm biomass and subsidized the green pathway increasing the contribution of autochthonous resources and energy fluxes, although a decrease in total energy fluxes were observed, which paralleled with a decrease in invertebrate density.
3. Treated effluent had complex effects on invertebrate diversity across spatial scales: they reduced taxa richness at local scale and increased community taxonomic heterogeneity.

General conclusions

1. The studied stressors altered the base of the food web, with diversion reducing the availability of coarse detritus and pollution consistently promoting biofilm biomass.

2. The manipulative experiment showed evident alterations of pollution on the organization of food webs, although the effects showed contrasting patterns to the observational experiment.
3. Indeterminacies regarding the responses of food webs to the same stressor may be given by changes in the baseline configuration of these food webs, for example by different interaction strengths between the different components of food webs, which were not directly measured in the current dissertation.

Ondorio orokorrak

Kutsadura eta ur-erauzketaren ondorio konbinatuak ibaietako sare trofikoetan:

1. Behaketa-esperimentuan kutsadura, ur-erauzketa eta haien arteko interakzioarekiko erantzun konplexuak behatu ziren sare trofikoetan, kutsadurak ur-erauzketarekiko erantzun gehienak modulatu zituelarik (1. eta 2. kapituluak).
2. Kutsadurak biofilmaren biomasa handitu zuen, eta, bide berdearen garrantzia murriztu bazuen ere, ekoizpen primarioan hautemandako emendioak sare trofikoen konplexutasuna handitu zuen, dibertsitate trofikoa handituz eta kate trofiko luzeagoak baimenduz.
3. Ur-erauzketak materia organikoaren metaketa murriztu zuen, zeinak bide arretik energiaren transferentzia urritu zuen eta ornogabeen dentsitatea murriztu, nahiz eta dibertsitate trofikoa zein heterogeneotasun taxonomikoa handitu ziren.
4. Kutsaduraren eta ur-erauzketaren arteko elkarreraginek are ondorio nabarmenagoak eragin zituen sare trofikoen konplexutasunean, bi estresoreen konbinazioak sare trofikoaren oinarrian aldaketarik eragin ez bazuen ere.

Kutsaduraren eragin nagusiak manipulazio-esperimentuan:

1. Manipulazio bidezko landa esperimentuak agerian utzi zuen tratamendu tertziarioa jasaten duten eta sisteman asko diluitzen diren efluenteek ere erantzun konplexuak sor ditzaketela sare trofikoen egitura eta funtzionamenduan. Erantzun hauek hain sentikorrak ez diren diseinu esperimentalen menpe hauteman ezinak lirateke. (3. eta 4. kapituluak).
2. Efluentearen adizioak biofilmaren biomasa emendatu eta bide berdea sustatu zuen, baliabide autoktonoen ekarpena handituz eta energia-fluxuak areagotuz, nahiz eta energia-fluxu totalak murriztu ziren ornogabeen dentsitatea urritzearekin batera.
3. Tratatutako efluenteak erantzun konplexuak eragin zituen ornogabeen dibertsitatean eskala espazial desberdinei dagokienez: maila lokalean taxonen aberastasuna murriztu zen eta eskala globalean komunitatearen heterogeneotasun taxonomikoa handitu zen.

Ondorio orokorrak

1. Aztertutako estresoreek sare trofikoaren oinarria aldatu zuten, ur-erauzketak detritu larrien eskuragarritasuna murriztu zuelarik eta kutsadurak biofilmaren biomasa handitu.
2. Esperimentu manipulatiboak kutsadurak sare trofikoen antolamenduan eragindako aldaketa nabarmenak erakutsi zituen, nahiz eta behaketa-esperimentuan estresore honek kontrako ondorioak eragin zituen.
3. Estresore berberak sare trofikoetan eragindako erantzun desberdinak sare trofiko horien jatorrizko ezaugarriak desberdinak izatearen ondorio izan daitezke, esaterako sare trofikoetako osagaien arteko interakzio-indarrak desberdinak izateagatik, zeinak lan honetan zuzenean neurtu ez ziren.

Conclusiones generales

Efectos combinados de la contaminación y la detracción de agua en las redes tróficas fluviales:

1. El experimento observacional mostró respuestas complejas de las redes tróficas frente a la contaminación, la detracción de agua y a su interacción, siendo la contaminación el factor que moduló la mayoría de las respuestas de la detracción de agua. (Capítulos 1 y 2)
2. La contaminación aumentó la biomasa del biofilm y, aunque disminuyó la importancia de la vía verde, los incrementos observados en la productividad primaria aumentaron la complejidad de las redes tróficas incrementando la diversidad trófica y permitiendo cadenas tróficas más largas.
3. La detracción de agua redujo el stock de materia orgánica, produciendo una disminución de la transferencia de energía a través de la vía marrón, una disminución de la densidad de invertebrados y un aumento tanto de la diversidad trófica como de la heterogeneidad taxonómica de la comunidad.
4. La interacción de la contaminación y la detracción de agua produjo efectos aún mayores en la complejidad de las redes tróficas, a pesar de que la combinación de ambos estresores no causó mayores alteraciones en la base de la red trófica.

Principales efectos de la contaminación en el experimento manipulativo:

1. El experimento manipulativo puso de manifiesto que incluso los efluentes con tratamiento terciario y altamente diluidos pueden generar respuestas complejas sobre la estructura y el funcionamiento de las redes tróficas, las cuales podrían haber pasado desapercibidas con diseños experimentales menos sensibles. (Capítulos 3 y 4).
2. La adición del efluente aumentó la biomasa del biofilm y promocionó la vía verde aumentando la contribución de los recursos autóctonos y los flujos de energía por esta vía, aunque se observó una disminución de los flujos totales de energía, que fue paralela a la disminución de la densidad de invertebrados.
3. El efluente tratado indujo efectos complejos sobre la diversidad de invertebrados a diferentes escalas espaciales: a escala local se redujo la riqueza de taxones y a escala global aumentó la heterogeneidad taxonómica de la comunidad.

Conclusiones generales

1. Los estresores estudiados alteraron la base de la red trófica, con la detracción reduciendo la disponibilidad de detritus grueso y la contaminación aumentando consistentemente la biomasa de biofilm.
2. El experimento manipulativo mostró evidentes alteraciones de la contaminación sobre la organización de las redes tróficas, aunque la contaminación en el experimento observacional produjo efectos opuestos.
3. Las inconsistencias en las respuestas de las redes tróficas a un mismo estresor pueden deberse a los cambios en la configuración de origen de estas redes tróficas, como las diferencias en las fuerzas de interacción entre los distintos componentes de las redes tróficas, las cuales no se midieron directamente en este trabajo.

Supplementary material

Chapter 1: Water diversion and pollution interactively shape freshwater food webs through bottom-up mechanisms

Tables

Table S1. Linear model results comparing the physicochemical and hydrological variables between rivers and reaches. The Pearson correlation between the variables in the PCA and the PC1 and TDN are shown. Asterisks indicate that the variables were log-transformed. Wet width was analyzed by means of mixed linear models with sampling campaign as random factor. Values in bold indicate statistical significance ($p < 0.05$).

	River		Reach		River : Reach		Correlation with PC1	Correlation with TDN
	F	P	F	P	F	P		
Cl^-(mg L^{-1})	5.68	**0.008**	0.89	0.360	0.88	0.473	**0.87**	**0.70**
SO_4^{-2} (mg L^{-1})*	32.11	**<0.001**	0.88	0.363	0.37	0.776	**0.93**	**0.67**
NO_3^- (mg L^{-1})*	1.57	0.236	0.83	0.377	0.54	0.659	0.33	0.20
NH_4^+ (µg N L-^{1})	15.29	**<0.001**	0.14	0.711	0.08	0.969	**0.57**	0.40
DOC (mg C L^{-1})	33.32	**<0.001**	3.92	0.065	0.40	0.756	**0.87**	**0.76**
TDN (mg N L^{-1})*	16.24	**<0.001**	0.08	0.783	0.18	0.910	**0.85**	
SRP (µg P L^{-1})*	8.74	**0.001**	0.00	0.996	0.22	0.881	**0.72**	**0.76**
pH	16.41	**<0.001**	0.00	0.960	0.42	0.741	**0.78**	**0.49**
Conductivity (µS cm^{-1})	185.88	**<0.001**	0.00	0.949	0.01	0.999	**0.97**	**0.82**
Temperature (°C)	1.51	0.251	0.02	0.880	0.03	0.993	**0.51**	**0.47**
Dissolved oxygen (%)*	2.59	0.094	0.07	0.799	1.29	0.311	-0.14	-0.04
Mean wet width (m)*	124.19	**<0.001**	52.10	**<0.001**	3.66	**0.013**		
Discharge (m^3 s^{-1})*	2.56	0.091	7.03	**0.017**	0.33	0.803		

Table S2. Mean and standard error of nitrogen and carbon stable isotope ratios for all the basal resources and invertebrate and fish taxa collected at each river and reach. The number of samples analyzed is shown in brackets. Rivers are ordered following $\log_{10}$(TDN) values from left to right.

		Urumea		Leitzaran		Kadagua		Deba	
		Control	Diverted	Control	Diverted	Control	Diverted	Control	Diverted
δ15N (‰)	Alder	-1.73 ± 0.07 (3)	-1.8 ± 0.06 (3)	-1.24 ± 0.11 (3)	-1.31 ± 0.13 (3)	-0.29 ± 0.27 (3)	-0.07 ± 0.27 (3)	-1.06 ± 0.14 (3)	-0.62 ± 0.4 (3)
	Biofilm	-0.18 ± 0.39 (3)	0.08 ± 0.19 (3)	4.08 ± 0.14 (3)	3.4 ± 0.44 (3)	6.03 ± 0.55 (3)	6.93 ± 0.77 (3)	6.9 ± 0.18 (3)	7.28 ± 1.12 (3)
	Bryophyte	0.89 ± 0.51 (3)	1.05 ± 0.22 (3)	6.84 ± 0.7 (2)	5.64 ± 0.21 (3)	-	-	-	2.94 ± 0.14 (3)
	Filamentous green algae	-	-	3.86 (1)	-	-	7.84 ± 1.07 (2)	6.1 ± 0.22 (3)	-
	Fine detritus	0.7 ± 0.19 (3)	0.56 ± 0.11 (2)	2.11 ± 0.04 (3)	2.58 ± 0.11 (3)	4.36 ± 0.44 (2)	4.51 ± 0.25 (3)	3.48 ± 0.19 (2)	2.3 ± 0.14 (3)
	Macrophyte	2.99 ± 0.41 (3)	-	-	-	9.41 ± 0.98 (2)	7.56 (1)	-	-
	Baetis	1.28 ± 0.02 (8)	1.01 ± 0.02 (9)	6.24 ± 0.07 (7)	5.86 ± 0.05 (9)	9.17 ± 0.09 (7)	9.52 ± 0.07 (4)	8.36 ± 0.21 (5)	8.18 ± 0.12 (8)
	Ecdyonurus	1.75 ± 0.1 (5)	1.2 ± 0.09 (5)	6.17 ± 0.08 (8)	5.68 ± 0.12 (9)	8.64 ± 0.08 (5)	9.03 ± 0.08 (5)	7.58 ± 0.29 (2)	8.57 (1)
	Echinogammarus	1.68 ± 0.08 (4)	1.34 ± 0.12 (4)	5.73 ± 0.06 (9)	5.64 ± 0.05 (9)	8.27 ± 0.04 (6)	8.43 ± 0.12 (3)	7.95 ± 0.13 (8)	7.41 ± 0.3 (4)
	Ephemerella	1.29 ± 0.05 (9)	0.8 ± 0.05 (7)	6.05 ± 0.07 (5)	6.04 ± 0.25 (2)	8.21 ± 0.17 (3)	9 ± 0.13 (6)	9.05 ± 0.17 (8)	8.02 ± 0.3 (9)
	Hydropsyche	2.42 ± 0.1 (6)	2.19 ± 0.12 (7)	6.99 ± 0.07 (9)	6.67 ± 0.09 (7)	8.62 ± 0.12 (9)	8.92 ± 0.04 (9)	9.63 ± 0.15 (9)	8.78 ± 0.4 (4)
	Rhyacophila	2.01 ± 0.2 (7)	2.29 ± 0.14 (9)	7.55 ± 0.21 (5)	7.25 ± 0.24 (5)	9.28 ± 0.16 (6)	9.35 ± 0.16 (7)	10.33 ± 0.13 (9)	10.29 ± 0.1 (6)
	Anguilla anguilla	6.4 ± 0.48 (4)	6.54 ± 0.19 (5)	-	-	14.65 ± 0.52 (5)	14.59 ± 0.28 (5)	13.49 ± 1.3 (5)	15.5 ± 0.36 (5)
	Barbatula quignardi	-	-	-	-	-	12.52 (1)	12.86 ± 1.11 (5)	13.57 ± 0.12 (5)
	Gobio lozanoi	-	-	-	-	-	14.22 ± 0.17 (4)	-	-
	Luciobarbus graellsii	-	-	-	-	14.65 ± 0.17 (5)	14.05 ± 0.22 (5)	-	-
	Parachondrostoma miegii	-	-	-	-	13.1 ± 0.25 (5)	13.23 ± 0.3 (5)	14.18 ± 0.44 (5)	13.45 ± 0.23 (5)
	Phoxinus bigerri	5.67 ± 0.21 (4)	5.68 ± 0.25 (4)	9.88 ± 0.1 (5)	9.04 ± 0.16 (5)	-	13.53 ± 0.21 (4)	13.29 ± 0.17 (5)	13.39 ± 0.65 (4)
	Salmo trutta	5.93 ± 0.29 (5)	5.81 ± 0.38 (5)	10.03 ± 0.24 (5)	9.41 ± 0.46 (5)	12.42 (1)	-	12.38 ± 0.12 (5)	11.69 ± 0.77 (5)

Table S2. *Continued.*

		Urumea		Leitzaran		Kadagua		Deba	
		Control	Diverted	Control	Diverted	Control	Diverted	Control	Diverted
δ13C (‰)	Alder	-29.52 ± 0.13 (3)	-29.56 ± 0.13 (3)	-30.03 ± 0.02 (3)	-30.18 ± 0.06 (3)	-30.54 ± 0.09 (3)	-29.81 ± 0.08 (3)	-29.9 ± 0.22 (3)	-29.75 ± 0.07 (3)
	Biofilm	-23.97 ± 0.81 (3)	-21.89 ± 1.04 (3)	-28.03 ± 0.77 (3)	-26.3 ± 1.14 (3)	-15.55 ± 0.62 (3)	-17.31 ± 0.96 (3)	-22.31 ± 1.47 (3)	-24.25 ± 1.85 (3)
	Bryophyte	-32.94 ± 1.03 (3)	-28.5 ± 0.98 (3)	-38.55 ± 0.75 (2)	-33.69 ± 1.15 (3)	-	-	-	-28.18 ± 1.09 (3)
	Filamentous green algae	-	-	-31.7 (1)	-	-	-28.29 ± 2.34 (2)	-28.61 ± 0.3 (3)	-
	Fine detritus	-27.06 ± 0.26 (3)	-27.58 ± 0.27 (2)	-27.81 ± 0.12 (3)	-27.65 ± 0.45 (3)	-15.8 ± 0.23 (2)	-17.71 ± 1.13 (3)	-21.75 ± 0.16 (2)	-20.34 ± 1.48 (3)
	Macrophyte	-25.52 ± 0.15 (3)	-	-	-	-24.24 ± 0.8 (2)	-26.49 (1)	-	-
	Baetis	-21.36 ± 0.31 (8)	-20.88 ± 0.15 (9)	-27.8 ± 0.13 (7)	-27.71 ± 0.08 (9)	-31.87 ± 0.17 (7)	-31.63 ± 0.27 (4)	-28.15 ± 0.17 (5)	-28.26 ± 0.06 (8)
	Ecdyonurus	-21.68 ± 0.74 (5)	-21.8 ± 0.48 (5)	-28.17 ± 0.15 (8)	-28.1 ± 0.09 (9)	-29.67 ± 0.34 (5)	-29.34 ± 0.29 (5)	-26.95 ± 0.2 (2)	-28.18 (1)
	Echinogammarus	-23.72 ± 0.19 (4)	-23.1 ± 0.3 (4)	-27.72 ± 0.12 (9)	-27.73 ± 0.11 (9)	-26.66 ± 0.07 (6)	-26.68 ± 0.16 (3)	-25.18 ± 0.13 (8)	-25.18 ± 0.21 (4)
	Ephemerella	-22.64 ± 0.26 (9)	-22.62 ± 0.12 (7)	-31.59 ± 0.37 (5)	-29.69 ± 0.76 (2)	-29.29 ± 0.22 (3)	-29.27 ± 0.17 (6)	-29.29 ± 0.06 (8)	-28.26 ± 0.14 (9)
	Hydropsyche	-24.21 ± 0.11 (6)	-23.61 ± 0.13 (7)	-29.32 ± 0.1 (9)	-28.48 ± 0.11 (7)	-28.01 ± 0.07 (9)	-28.2 ± 0.1 (9)	-26.34 ± 0.1 (9)	-26.25 ± 0.13 (4)
	Rhyacophila	-21.56 ± 0.69 (7)	-21.78 ± 0.52 (9)	-28.24 ± 0.49 (5)	-27.87 ± 0.27 (5)	-28.18 ± 0.17 (6)	-28.28 ± 0.1 (7)	-25.91 ± 0.11 (9)	-26.01 ± 0.07 (6)
	Anguilla anguilla	-22.89 ± 0.27 (4)	-21.84 ± 0.18 (5)	-	-	-26.42 ± 0.63 (5)	-26.03 ± 0.47 (5)	-24.42 ± 0.46 (5)	-24.53 ± 0.2 (5)
	Barbatula quignardi	-	-	-	-	-	-26.67 (1)	-24.27 ± 0.4 (5)	-24.95 ± 0.25 (5)
	Gobio lozanoi	-	-	-	-	-	-25.53 ± 0.09 (4)	-	-
	Luciobarbus graellsii	-	-	-	-	-25.2 ± 0.32 (5)	-25.07 ± 0.53 (5)	-	-
	Parachondrostoma miegii	-	-	-	-	-25.52 ± 0.27 (5)	-25.44 ± 0.08 (5)	-24.54 ± 0.15 (5)	-24.57 ± 0.1 (5)
	Phoxinus bigerri	-24.1 ± 0.45 (4)	-20.59 ± 0.38 (4)	-26.28 ± 0.14 (5)	-25.39 ± 0.26 (5)	-	-26.22 ± 0.15 (4)	-24.15 ± 0.2 (5)	-24.27 ± 0.31 (4)
	Salmo trutta	-23.09 ± 0.42 (5)	-21.64 ± 0.5 (5)	-25.77 ± 0.16 (5)	-25.83 ± 0.32 (5)	-27.34 (1)	-	-24.82 ± 0.15 (5)	-24.98 ± 0.36 (5)

Table S3. Trophic position (mean and standard error) of fish species in each reach. Values in bold belong to the species showing the maximum FCL at each reach. The number of individuals is shown in brackets. Rivers are ordered following $\log_{10}$(TDN) values from left to right.

Taxon	Urumea		Leitzaran		Kagadua		Deba	
	Control	Diverted	Control	Diverted	Control	Diverted	Control	Diverted
Anguilla anguilla	**1.33 ± 0.11 (4)**	**1.49 ± 0.04 (5)**			2.220 ± 0.12 (5)	**2.10 ± 0.06 (5)**	2.19 ± 0.30 (5)	**2.85 ± 0.08 (5)**
Barbatula quignardi						1.63 (1)	2.05 ± 0.25 (5)	2.41 ± 0.03 (5)
Gobio lozanoi						2.01 ± 0.04 (4)		
Luciobarbus graellsii					**2.221 ± 0.04 (5)**	1.98 ± 0.05 (5)		
Parachondrostoma miegii					1.87 ± 0.06 (5)	1.79 ± 0.07 (5)	**2.35 ± 0.10 (5)**	2.38 ± 0.05 (5)
Phoxinus bigerri	1.17 ± 0.05 (4)	1.3 ± 0.06 (4)	1.53 ± 0.02 (5)	1.47 ± 0.04 (5)		1.86 ± 0.05 (4)	2.14 ± 0.04 (5)	2.37 ± 0.15 (4)
Salmo trutta	1.23 ± 0.07 (5)	1.33 ± 0.09 (5)	**1.57 ± 0.05 (5)**	**1.55 ± 0.11 (5)**	1.71 (1)		1.94 ± 0.03 (5)	1.98 ± 0.18 (5)

Table S4. Mean and standard error of the biomass of basal resources in each reach of the studied rivers. Rivers are ordered following log10(TDN) values from left to right.

	Urumea		Leitzaran		Kadagua		Deba	
	Control	Diverted	Control	Diverted	Control	Diverted	Control	Diverted
Coarse detritus (g m^{-2})	9.64 ± 1.68	17.21 ± 9.39	20.95 ± 2.37	10.63 ± 2.08	5.13 ± 1.84	0.91 ± 0.18	17.35 ± 3.62	8.38 ± 2.44
Fine detritus (g m^{-2})	23.89 ± 3.01	21.18 ± 6.68	45.94 ± 6.35	42.62 ± 5.34	24.89 ± 3.83	73.68 ± 17.88	25.64 ± 7.84	38.84 ± 5.05
Biofilm (mg m^{-2})	10.81 ± 1.52	7.16 ± 1.14	22.74 ± 3.74	33.55 ± 6.73	46.49 ± 3.39	47.05 ± 2.63	27.09 ± 6.21	24.26 ± 3.18

Table S5. Contribution of a) Alder, b) fine detritus and c) autochthonous resources (biofilm, filamentous green algae, bryophytes and macrophytes) to the diets of consumers in each sampling reach. Lower and upper CI indicate the lower and upper limit of the 95% credibility intervals. Mode indicates the global mode. Rivers are ordered following $\log_{10}$(TDN) values from left to right.

		Urumea		Leitzaran		Kadagua		Deba	
		Control	Diverted	Control	Diverted	Control	Diverted	Control	Diverted
Alder contribution	Mean	0.01	0.21	0.02	0.03	0.41	0.08	0.01	0.04
	Median	0.01	0.21	0.02	0.02	0.41	0.07	0.01	0.04
	Mode	0.001	0.12	0.02	0.01	0.41	0.05	0.01	0.01
	Lower CI	0	0.07	0	0	0.31	0	0	0
	Upper CI	0.03	0.38	0.04	0.07	0.5	0.16	0.03	0.12
Fine detritus contribution	Mean	0.04	0.06	0.12	0.44	0.08	0.23	0.15	0.27
	Median	0.05	0.06	0.11	0.46	0.08	0.21	0.16	0.26
	Mode	0.05	0.01	0.1	0.5	0.06	0.16	0.16	0.25
	Lower CI	0	0	0.03	0.18	0.02	0	0.04	0.14
	Upper CI	0.07	0.12	0.23	0.66	0.16	0.5	0.25	0.41
Autochthonous resource contribution	Mean	0.94	0.73	0.86	0.53	0.51	0.69	0.84	0.68
	Median	0.94	0.73	0.86	0.52	0.51	0.7	0.83	0.69
	Mode	0.95	0.66	0.87	0.47	0.51	0.75	0.83	0.72
	Lower CI	0.91	0.56	0.77	0.33	0.43	0.43	0.75	0.53
	Upper CI	0.99	0.9	0.94	0.78	0.59	0.95	0.94	0.83

Table S6. Linear mixed model results of stable isotopes for the trophic compartments and the whole community with sample as random factor and TDN and reach as fixed factors. Values in bold indicate significant differences between factors ($p < 0.05$). Asterisks indicate that predatory invertebrate was analyzed with linear models as we only had one taxon.

Stable Isotope	Trophic compartments	TDN		Reach		TDN : Reach		Coefficients	
		F	P	F	P	F	P	TDN	TDN : Reach (D)
δ15N	Entire community	1620.99	**<0.001**	0.92	0.338	0.33	0.564	19.094	
	Resources	66.65	**<0.001**	0.02	0.892	0.85	0.358	11.896	
	Primary consumers	1282.15	**<0.001**	2.01	0.158	0.53	0.467	20.510	
	Predatory invertebrate*	544.01	**<0.001**	0.14	0.712	0.13	0.724	23.111	
	Fish	414.16	**<0.001**	0.13	0.718	0.91	0.343	19.723	
δ13C	Entire community	79.39	**<0.001**	3.32	0.069	4.34	**0.038**	-6.101	-3.552
	Resources	10.23	**0.002**	1.15	0.286	0.44	0.510	10.239	
	Primary consumers	149.71	**<0.001**	0.43	0.512	1.67	0.198	-20.700	
	Predatory invertebrate*	31.02	**<0.001**	0.11	0.746	0.45	0.504	-11.383	
	Fish	35.55	**<0.001**	1.57	0.212	12.80	**0.001**	-2.033	-6.271

Table S7. Results of 2-way ANOVA of stable isotopes for all the taxa analyzed with TDN and reach as factors. Values in bold indicate significant differences between factors ($p < 0.05$).

Stable Isotope	Taxon	TDN		Reach		TDN : Reach		Coefficients		
		F	P	F	P	F	P	TDN	Reach (D)	TDN : Reach (D)
δ15N	Alder	15.92	**0.001**	0.38	0.542	0.83	0.372	2.591		
	Biofilm	124.90	**<0.001**	0.19	0.669	0.16	0.694	20.258		
	Bryophyte	3.45	0.093	0.71	0.418	9.73	**0.011**			-39.545
	Fine detritus	16.31	**0.001**	0.01	0.922	1.12	0.306	9.471		
	Filament	1.03	0.385	11.47	**0.043**				2.891	
	Macrophyte	52.13	**0.005**	2.36	0.222			26.609		
	Baetis	252.74	**<0.001**	1.81	0.184	0.36	0.551	22.936		
	Ecdyonurus	242.38	**<0.001**	0.00	0.954	4.78	**0.035**	21.140		6.847
	Echinogammarus	162.66	**<0.001**	0.35	0.555	0.11	0.746	16.665		
	Ephemerella	299.19	**<0.001**	0.72	0.402	0.12	0.734	22.138		
	Hydropsyche	407.84	**<0.001**	0.24	0.624	0.88	0.353	19.443		
	Rhyacophila	544.01	**<0.001**	0.14	0.712	0.13	0.724	23.111		
	Anguilla anguilla	95.06	**<0.001**	0.87	0.360	0.93	0.344	21.858		
	Barbatula quignardi	0.14	0.718	0.40	0.544					
	Luciobarbus graellsii			4.56	0.065					
	Parachondrostoma miegii	4.25	0.056	0.91	0.356	1.84	0.194			
	Phoxinus bigerri	248.70	**<0.001**	0.71	0.407	1.21	0.280	20.792		
	Salmo trutta	131.79	**<0.001**	1.82	0.188	0.40	0.535	18.032		
δ13C	Alder	2.67	0.118	1.52	0.232	1.23	0.280			
	Biofilm	1.17	0.292	0.00	0.989	0.88	0.359			
	Bryophyte	0.97	0.348	8.92	**0.014**	4.64	0.057		10.52	
	Fine detritus	16.40	**0.001**	0.00	0.987	0.09	0.773	23.723		
	Filament	1.15	0.362	1.12	0.368					
	Macrophyte	0.91	0.410	7.14	0.076					
	Baetis	88.16	**<0.001**	1.39	0.243	0.34	0.561	-24.972		
	Ecdyonurus	59.18	**<0.001**	0.20	0.654	0.57	0.455	-21.342		
	Echinogammarus	0.55	0.462	0.01	0.920	0.78	0.381			
	Ephemerella	56.48	**<0.001**	2.15	0.149	0.18	0.671	-17.856		
	Hydropsyche	11.02	**0.002**	0.26	0.613	3.42	0.070	-3.012		
	Rhyacophila	31.02	**<0.001**	0.11	0.746	0.45	0.504	-11.383		
	Anguilla anguilla	15.85	**0.001**	0.69	0.415	0.88	0.358	-5.611		
	Barbatula quignardi	6.99	**0.030**	2.08	0.187			15.516		
	Luciobarbus graellsii			0.04	0.838					
	Parachondrostoma miegii	31.33	**<0.001**	0.01	0.915	0.11	0.915	8.830		
	Phoxinus bigerri	4.49	**0.043**	1.62	0.214	7.90	**0.009**	1.258		-11.872
	Salmo trutta	12.06	**0.002**	1.05	0.314	0.95	0.339	-4.661		

Table S8. Bayesian community wide metrics of the iso-space for consumers in each sampling reach. Lower and upper CI indicate the lower and upper limit of the 95% credibility intervals. Rivers are ordered following $\log_{10}$(TDN) values from left to right.

		Urumea		Leitzaran		Kadagua		Deba	
		Control	Diverted	Control	Diverted	Control	Diverted	Control	Diverted
CD	Mean	2	1.89	1.77	1.7	2.44	2.45	1.9	2.22
	Median	2	1.89	1.76	1.7	2.44	2.45	1.89	2.21
	Mode	2	1.88	1.76	1.7	2.4	2.41	1.89	2.18
	Lower CI	1.66	1.62	1.46	1.4	2.17	2.22	1.7	1.96
	Upper CI	2.35	2.15	2.13	1.98	2.69	2.65	2.11	2.47
SEA	Mean	8.48	8.12	6.43	3.84	14.08	10.29	11.35	9.79
	Median	8.37	8.02	6.37	3.79	13.89	10.2	11.23	9.68
	Mode	7.96	7.94	6.37	3.61	13.74	9.93	11.18	9.53
	Lower CI	6.3	5.98	4.84	2.81	10.29	7.62	8.6	7.42
	Upper CI	10.82	10.26	8.3	4.95	18.06	12.97	14.2	12.5
MNND	Mean	1.92	2	2.04	1.82	2.7	2.42	2.54	2.9
	Median	1.91	1.98	2	1.79	2.67	2.41	2.53	2.89
	Mode	1.9	1.93	1.96	1.75	2.61	2.38	2.54	2.86
	Lower CI	1.51	1.55	1.48	1.36	2.29	2.06	2.23	2.49
	Upper CI	2.35	2.4	2.7	2.34	3.2	2.78	2.85	3.29
SDNND	Mean	1.94	1.51	1.15	1.34	1.72	2.26	0.49	0.7
	Median	1.94	1.52	1.15	1.35	1.73	2.28	0.46	0.68
	Mode	1.88	1.57	1.14	1.29	1.79	2.25	0.36	0.67
	Lower CI	1.3	0.61	0.25	0.45	0.84	1.56	0	0
	Upper CI	2.56	2.5	2.04	2.24	2.53	2.91	1.05	1.33

Figures

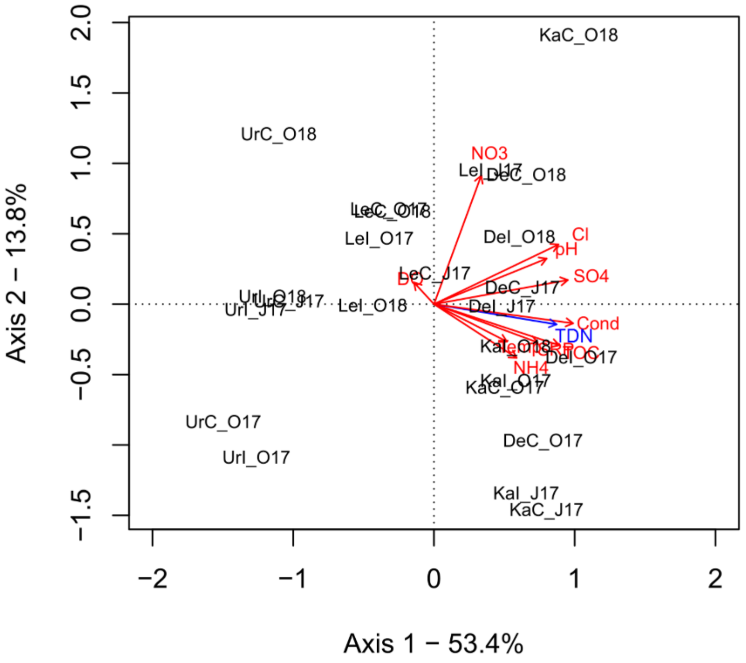

Figure S1. PCA ordination of the studied rivers at each sampling date according to the water physicochemical variables. Percentage of variation explained by each axis is shown.

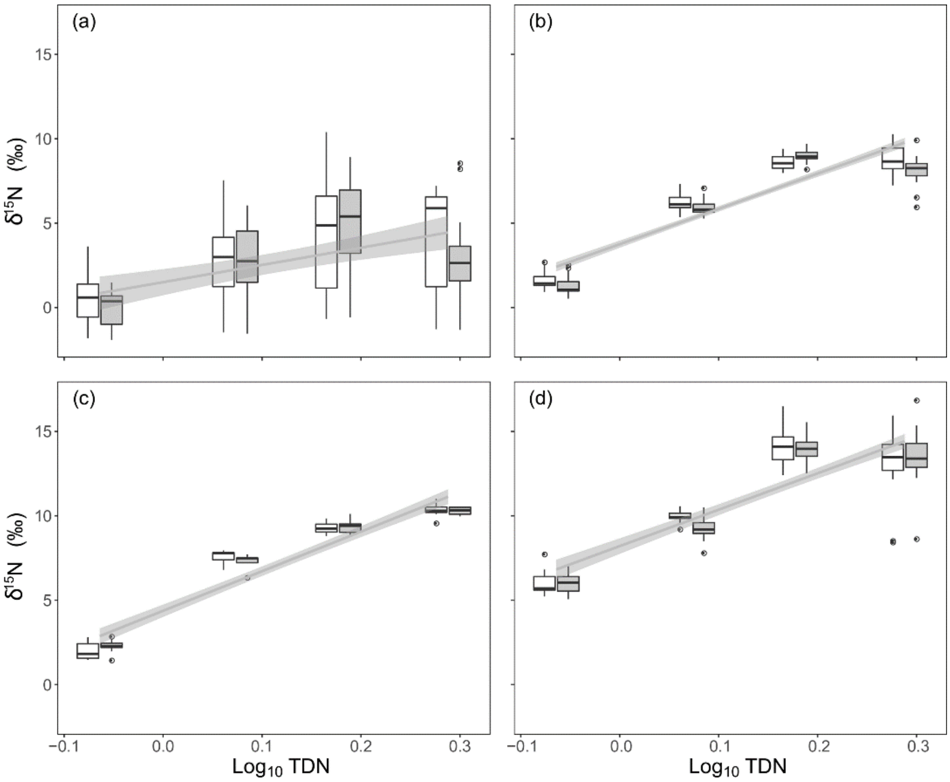

Figure S2. Nitrogen stable isotope ratios ($\delta^{15}N$, ‰) of (a) basal resources, (b) primary consumers, (c) predatory invertebrates, and (d) fish along the total dissolved nitrogen (TDN) gradient (white boxes for control; grey for diverted). The box plots show the median, the interquartile range and the tails of the distribution. A single grey regression line was represented as only the TDN gradient was significant.

Chapter 2: Pollution modulates the effects of water diversion on stream food web energy fluxes

Tables

Table S1. Linear model results comparing mean BM, Biomass and MR between reaches along the pollution gradient. Bold values indicate statistical significance ($p < 0.05$). Coefficients are shown for significant responses and pollution gradient (Log$_{10}$TDN) and C reaches as reference in all cases.

		Log$_{10}$TDN			Reach			Log$_{10}$TDN:Reach		
		F	p	Coeff.	F	p	Coeff.	F	p	Coeff.
Primary consumers	Mean BM	4.35	**0.037**	0.62	1.57	0.211		5.29	**0.022**	-0.67
	Biomass	0.65	0.422		3.74	0.054		16.8	**<0.001**	-2.08
	MR	0.78	0.377		5.93	**0.015**	-0.14	15.96	**<0.001**	-1.8
Omnivores	Mean BM	5.74	**0.017**	0.95	0.06	0.812		0.02	0.886	
	Biomass	13.32	**<0.001**	1.59	0.41	0.52		3.54	0.06	
	MR	18.73	**<0.001**	1.44	0.71	0.398		4.16	**0.042**	-0.95
Carnivores	Mean BM	2.33	0.128		0	0.98		0.12	0.731	
	Biomass	3.78	0.053		6.56	**0.011**	0.36	3.07	0.081	
	MR	4.7	**0.031**	1.75	9.08	**0.003**	0.33	4.58	**0.033**	-1.7

Table S2 AFDM in grams (mean and standard error) and abundance of fish sampled in the study. Rivers are ordered following $\log_{10}$(TDN) values from left to right.

	Urumea		Leitzaran		Kadagua		Deba	
Etiquetas de fila	Control	Impact	Control	Impact	Control	Impact	Control	Impact
Anguilla anguilla	4.79 ± 1.69 (31)	20.52 ± 4.62 (46)	0.17 (1)		15.51 ± 1.92 (68)	8.32 ± 0.84 (153)	43.61 ± 7.44 (29)	36.52 ± 12.76 (8)
Barbatula quignardi					0.05 (1)	0.1 ± 0.03 (4)	0.66 ± 0.06 (9)	0.74 ± 0.02 (112)
Gobio lozanoi						4.63 ± 0.25 (4)		
Luciobarbus graellsii					25.03 ± 10.14 (11)	48.5 ± 10.54 (23)		
Parachondrostoma miegii					12.27 ± 0.78 (33)	9.04 ± 1.12 (15)	4.16 ± 0.28 (67)	2.82 ± 0.13 (168)
Phoxinus bigerri	0.28 ± 0.01 (260)	0.35 ± 0.03 (57)	0.58 ± 0.03 (202)	0.75 ± 0.02 (232)	0.04 ± 0.004 (9)	0.67 ± 0.2 (28)	0.36 ± 0.02 (58)	0.42 ± 0.01 (306)
Salmo salar	0.97 (1)	1.56 ± 0.64 (2)						
Salmo trutta	2.65 ± 0.81 (63)	4.57 ± 1.42 (40)	32.42 ± 6.35 (39)	13.12 ± 1.82 (63)	1.13 (1)		4.09 ± 2.5 (27)	4.76 ± 3.05 (10)
Density (ind/m^2)	0.21	0.29	0.30	0.29	0.29	0.59	0.19	0.46
g/m^2	0.23	2.27	1.71	0.99	4.07	6.65	1.66	0.78
AFDM/ind	1.10	7.93	5.71	3.39	14.12	11.29	8.85	1.70

Table S3. Linear model results comparing energy fluxes regarding functions for each trophic group between reaches along the pollution gradient. Bold values indicate statistical significance ($p < 0.05$). Coefficients are shown for significant responses and pollution gradient ($Log_{10}TDN$) and C reaches as reference in all cases.

		$Log_{10}TDN$			Reach			$Log_{10}TDN$:Reach		
		F	p	Coeff.	F	p	Coeff.	F	p	Coeff.
Herbivory	Primary consumers	0.12	0.725		0.12	0.733		5.07	**0.028**	-3.89
	Omnivores	12.47	**0.001**	5.28	0.01	0.906		4.14	**0.046**	-3.86
	Carnivores	-	-	-	-	-	-	-	-	-
Detritivory	Primary consumers	0.59	0.445		8.7	**0.004**	-0.49	17.35	<0.001	-5.35
	Omnivores	0.83	0.365		0.96	0.33		9.88	**0.002**	-5.29
	Carnivores	-	-	-	-	-	-	-	-	-
Carnivory	Primary consumers	-	-	-	-	-	-	-	-	-
	Omnivores	6.57	**0.013**	3.82	0.74	0.393		1.11	0.297	
	Carnivores	1.27	0.264		1.53	0.221		32.85	**<0.001**	-8.16

Figures

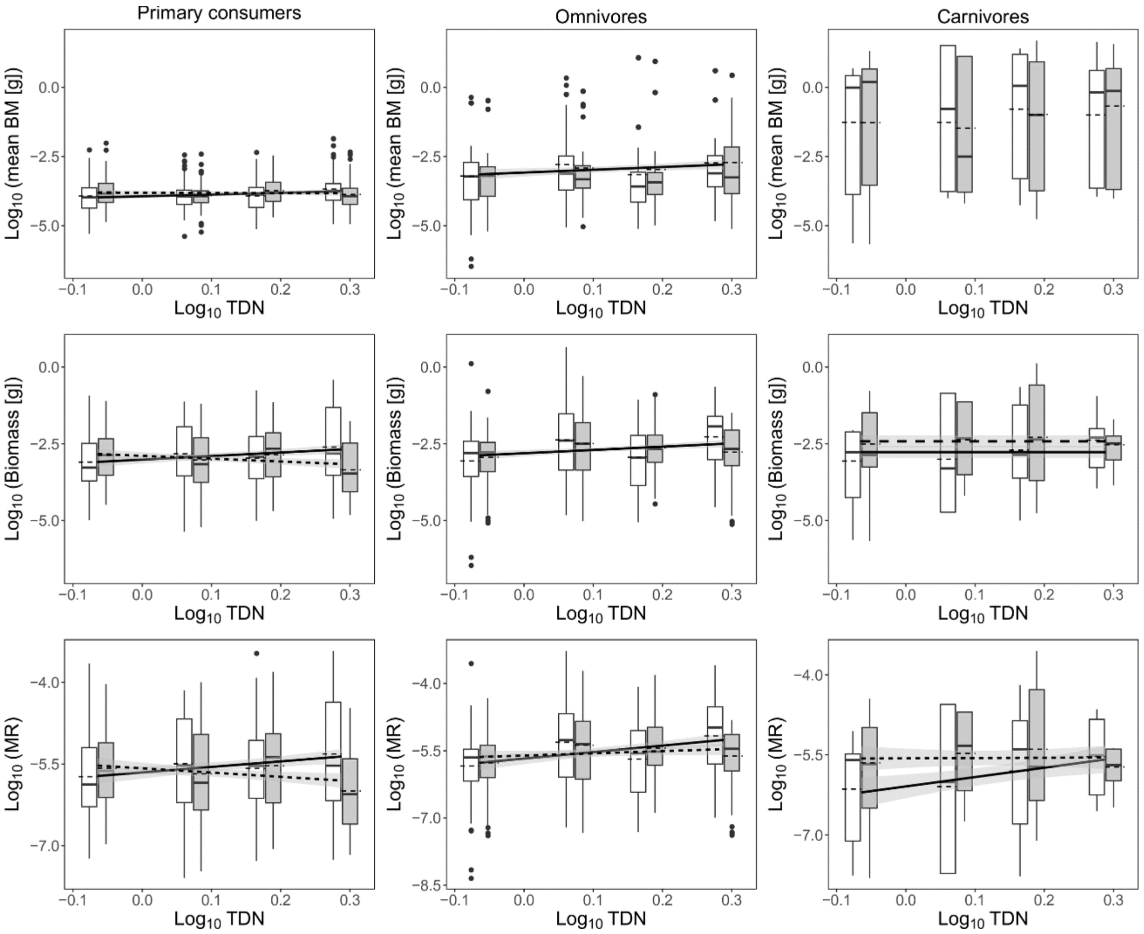

Fig. S1. Mean body mas, biomass and metabolic rate (MR) of primary consumers, omnivores and carnivores respectively. The box plots show the median, the interquartile range and the tails of the distribution. Dashed lines represent the mean value. White box plots refer to control reaches, grey box plots to impact. A single black regression line is represented when only the TDN gradient was significant and black regression lines (solid line for control; dashed line for diverted) are drawn when the effect of the diversion differed. Bands around the line represent the 95 % confidence interval.

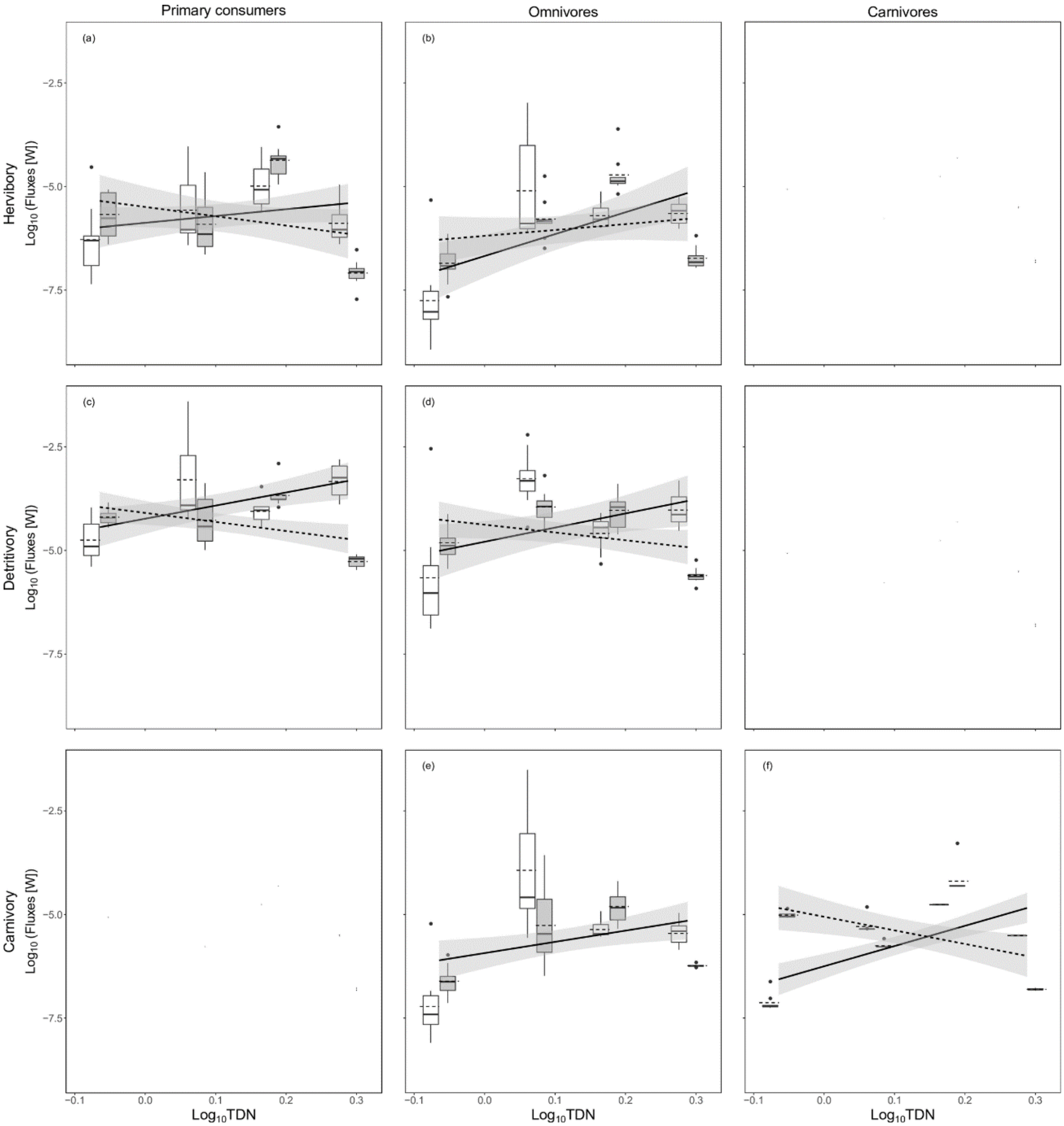

Fig. S2. Energy fluxes to each trophic guild (primary consumers, omnivores, and carnivores) and function (herbivory, detritivory and carnivory). The box plots show the median, the interquartile range and the tails of the distribution. Dashed lines represent the mean value. White box plots refer to control reaches, grey box plots to impact. A single black regression line is represented when only the TDN gradient was significant and black regression lines (solid line for control; dashed line for diverted) are drawn when the effect of the diversion differed. Bands around the line represent the 95 % confidence interval.

Chapter 3: Treated and highly diluted wastewater promotes the green food web but reduces trophic diversity

Tables

Table S1. Physicochemical properties of the effluent and its contribution to the stream during the two previous months before each sampling occasion of the After period. BOD$_5$ and COD are the Biochemical and Chemical Oxygen Demand, TP is Total Phosphorous and TN is Total Nitrogen. Asterisks indicate the logarithmized parameters in linear models.

	Aug'17-Oct'17	Nov'17-Jan'18	Mar'18-May'18	F	p
pH	6.95 ± 0.05 (28)	7.12 ± 0.05 (30)	6.96 ± 0.06 (25)	3.89	**0.024**
Conductivity (µS/cm)	678.8 ± 19.9 (28)	446.8 ± 13.4 (31)	610.9 ± 26.6 (25)	38.30	**<0.001**
BOD$_5$* (mg/l O$_2$)	8.29 ± 0.60 (28)	7.03 ± 0.63 (31)	10.20 ± 0.97 (25)	4.75	**0.011**
COD* (mg/l O$_2$)	39.61 ± 2.40 (28)	28.55 ± 1.66 (31)	39.32 ± 4.29 (25)	5.35	**0.007**
TP* (mg/l PO$_4^{3-}$)	1.38 ± 0.19 (10)	1.34 ± 0.14 (9)	1.61 ± 0.25 (10)	0.27	0.765
TN (mg/l NO$_3^-$)	6.26 ± 0.57 (28)	4.15 ± 0.63 (30)	4.02 ± 1.32 (23)	11.17	**<0.001**
Effluent contribution (%)	3.64± 0.03 (61)	1.22 ± 0.07 (61)	1.91 ± 0.09 (61)	342.91	**<0.001**

Table S2. Trophic position (mean and standard error) of fish species in each reach. Values in bold belong to the species showing the maximum FCL at each reach. The number of individuals is shown in brackets. Periods Before (B) and After (A), Seasons Autumn (Au), Winter (W) and Spring (S), and Control (C) and Impact (I) Reaches are indicated.

			Anguilla anguilla	*Barbatula quignardi*	*Parachondrostoma arrigonis*	*Phoxinus bigerri*	*Salmo trutta*
B	Au	C	3.11 ± 0.26 (4)			**3.24 ± 0.08 (5)**	3.13 ± 0.12 (5)
		I	3.45 ± 0.14 (5)	3.93 ± 0.12 (5)		**4.61 ± 0.26 (5)**	3.65 ± 0.16 (5)
	W	C	3.94 ± 0.18 (3)			**4.14 ± 0.20 (5)**	4.09 ± 0.1 (2)
		I	3.92 ± 0.23 (5)	4.03 ± 0.04 (5)		**4.82 ± 0.16 (4)**	3.83 ± 0.26 (5)
	S	C	3.74 ± 0.15 (5)			**3.90 ± 0.10 (5)**	3.47 ± 0.01 (2)
		I	3.66 ± 0.03 (5)	3.75 ± 0.09 (3)		**4.43 ± 0.11 (5)**	3.34 ± 0.11 (8)
A	Au	C	2.76 ± 0.18 (4)			3.65 ± 0.11 (4)	**3.73 ± 0.06 (4)**
		I	3.14 ± 0.32 (4)	3.74 ± 0.06 (5)		**4.29 ± 0.09 (5)**	3.97 ± 0.06 (4)
	W	C				3.75 ± 0.40 (5)	**3.93 (1)**
		I	4.53 ± 0.07 (5)	4.15 ± 0.10 (5)	**4.65 ± 0.12 (2)**	4.43 ± 0.12 (5)	3.94 (1)
	S	C	**3.39 ± 0.20 (4)**			3.02 ± 0.06 (5)	2.88 ± 0.02 (4)
		I	3.79 ± 0.18 (5)	**3.798 (1)**		3.78 ± 0.08 (5)	3.24 ± 0.15 (5)

Table S3. Model-selection to explain the effect of the effluent addition on basal resource (coarse detritus, fine detritus and autochthonous resources) contribution to primary consumers. Degrees of freedom (df), log-likelihood ratios (logLik), Bayesian Information Criterion (BIC), and the difference with the model with lowest value (ΔBIC) are given. Models with the lowest BIC are shown in bold. Coefficients related to the effect of the effluent are shown for the best model. Period (P), Reach (R) and Season (S) are included as factors.

	Model	P	R	S	P:R	P:S	R:S	P:R :S	df	logLik	BIC	ΔBIC	P(A):R(I)	P(A):R(I):S(W)	P (A):R (I):S (Sp)
Coarse detritus contribution	**glm0**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**12**	**-59242.56**	**118624.2**	**0**	**-0.54**	**0.24**	**1.03**
	glm2	+	+	+		+	+		9	-60023.27	120150.8	1526.64			
	glm1	+	+	+	+	+	+		10	-60039.27	120194.4	1570.24			
	glm10	+		+		+			6	-60518.04	121105.6	2481.41			
	glm7	+	+	+		+			7	-60519.58	121120.3	2496.08			
	glm4	+	+	+	+	+			8	-60536.61	121165.9	2541.73			
	glm5	+	+	+			+		7	-62031.7	124144.5	5520.33			
	glm3	+	+	+	+		+		8	-62045.91	124184.5	5560.33			
	glm8		+	+			+		6	-62293.57	124656.7	6032.47			
	glm13	+		+					4	-62519.9	125086.2	6461.95			
	glm6	+	+	+	+				6	-62538.71	125147	6522.76			
	glm16			+					3	-62781.08	125596.9	6972.73			
	glm11		+	+					4	-62782.62	125611.6	6987.39			
	glm14	+							2	-73768.1	147559.4	28935.18			
	glm12	+	+						3	-73769.58	147573.9	28949.73			
	glm9	+	+		+				4	-73787.78	147621.9	28997.72			
	glm17								1	-74011.72	148035	29410.83			
	glm15		+						2	-74013.2	148049.6	29425.38			

Table S3. *Continued*

	Model	P	R	S	P:R	P:S	R:S	P:R :S	df	logLik	BIC	ΔBIC	P(A):R(I)	P(A):R(I):S(W)	P (A):R (I):S (Sp)
Fine detritus contribution	**glm17**								**1**	**-17906.6**	**35824.8**	**0**			
	glm15		+						2	-17906.29	35835.8	10.96			
	glm14	+							2	-17908.93	35841	16.25			
	glm12	+	+						3	-17908.62	35852	27.21			
	glm9	+	+		+				4	-17910.58	35867.5	42.72			
	glm16			+					3	-17947.69	35930.2	105.37			
	glm11		+	+					4	-17947.39	35941.1	116.35			
	glm13	+		+					4	-17950.07	35946.5	121.71			
	glm8		+	+			+		6	-17949.66	35968.9	144.08			
	glm10	+		+		+			6	-17951.25	35972	147.26			
	glm6	+	+	+	+				6	-17951.77	35973.1	148.29			
	glm7	+	+	+		+			7	-17950.95	35983	158.24			
	glm5	+	+	+			+		7	-17952.04	35985.2	160.43			
	glm4	+	+	+	+	+			8	-17952.96	35998.6	173.84			
	glm3	+	+	+	+		+		8	-17954.03	36000.8	175.98			
	glm2	+	+	+		+	+		9	-17953.23	36010.8	185.99			
	glm1	+	+	+	+	+	+		10	-17955.24	36026.4	201.58			
	glm0	+	+	+	+	+	+	+	12	-17955.06	36049.2	224.41			

Table S3. *Continued*

	Model	P	R	S	P:R	P:S	R:S	P:R:S	df	logLik	BIC	ΔBIC	P(A):R(I)	P(A):R(I):S(W)	P (A):R (I):S (Sp)
Autochthonous resource contribution	**glm0**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**12**	**-48142.48**	**96424**	**0**	**0.44**	**0.09**	**-0.86**
	glm4	+	+	+	+	+			8	-48358.15	96809	384.98			
	glm1	+	+	+	+	+	+		10	-48353.25	96822.4	398.37			
	glm10	+		+		+			6	-48415.63	96900.8	476.75			
	glm7	+	+	+		+			7	-48431.68	96944.5	520.45			
	glm2	+	+	+		+	+		9	-48437.04	96978.4	554.34			
	glm6	+	+	+	+				6	-48619.82	97309.2	885.13			
	glm3	+	+	+	+		+		8	-48624.85	97342.4	918.39			
	glm13	+		+					4	-48677.04	97400.4	976.4			
	glm5	+	+	+			+		7	-48699.07	97479.3	1055.22			
	glm16			+					3	-48788.58	97611.9	1187.88			
	glm11		+	+					4	-48804.57	97655.5	1231.45			
	glm8		+	+			+		6	-48810.66	97690.9	1266.83			
	glm9	+	+		+				4	-51705.65	103457.7	7033.62			
	glm14	+							2	-51760.77	103544.7	7120.68			
	glm12	+	+						3	-51776.47	103587.7	7163.67			
	glm17								1	-51868.86	103749.3	7325.26			
	glm15		+						2	-51884.55	103792.3	7368.25			

Table S4. Model-selection to explain the effect of th effluent addition on the iso-space metrics of the consumers (invertebrates and fish). Degrees of freedom (df), log-likelihood ratios (logLik), Bayesian Information Criterion (BIC), and the difference with the model with lowest value (ΔBIC) are given. Models with the lowest BIC are shown in bold. Coefficients related to the effect of the effluent are shown for the best model. Period (P), Reach (R) and Season (S) are included as factors.

	Model	P	R	S	P:R	P:S	R:S	P:R:S	df	logLik	BIC	ΔBIC	P (A):R (I)	P (A):R (I):S (W)	P (A):R (I):S (S)
CD	**glm0**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**13**	**33254.16**	**-66368.2**	**0**	-0.9	0.66	1.04
	glm1	+	+	+	+	+	+		11	19381.2	-38643.8	27724.36			
	glm2	+	+	+		+	+		10	13534.2	-26960.6	39407.58			
	glm3	+	+	+	+		+		9	13340.32	-26583.6	39784.56			
	glm5	+	+	+			+		8	8676.76	-17267.3	49100.9			
	glm4	+	+	+	+	+			9	8466.31	-16835.6	49532.57			
	glm7	+	+	+		+			8	4594.66	-9103.1	57265.1			
	glm6	+	+	+	+				7	4460.9	-8846.3	57521.84			
	glm8		+	+			+		7	3558.06	-7040.7	59327.51			
	glm10	+		+		+			7	2851.45	-5627.4	60740.74			
	glm13	+		+					5	-371.82	797.5	67165.73			
	glm11		+	+					5	-2698.61	5451.1	71819.31			
	glm16			+					4	-3997.1	8037.3	74405.51			
	glm9	+	+		+				5	-35440.11	70934.1	137302.29			
	glm12	+	+						4	-36105.15	72253.4	138621.61			
	glm14	+							3	-36434.57	72901.5	139269.66			
	glm15		+						3	-36972.26	73976.8	140345.03			
	glm17								2	-37290.06	74601.7	140969.86			

Table S4.*Continued.*

	Model	P	R	S	P:R	P:S	R:S	P:R:S	df	logLik	BIC	ΔBIC	P (A):R (I)	P (A):R (I):S (W)	P (A):R (I):S (S)
SEA	**glm0**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**13**	**-55544.76**	**111229.7**	**0**	-0.42	1.82	-0.61
	glm2	+	+	+		+	+		10	-58101.27	116310.3	5080.68			
	glm1	+	+	+	+	+	+		11	-58100.47	116319.5	5089.85			
	glm5	+	+	+			+		8	-74853.82	149793.9	38564.22			
	glm3	+	+	+	+		+		9	-74853.42	149803.9	38574.2			
	glm7	+	+	+		+			8	-76902.77	153891.8	42662.11			
	glm4	+	+	+	+	+			9	-76902.4	153901.8	42672.16			
	glm10	+		+		+			7	-76959.83	153995.1	42765.45			
	glm8		+	+			+		7	-79164.14	158403.7	47174.07			
	glm6	+	+	+	+				7	-86006.65	172088.7	60859.09			
	glm13	+		+					5	-86045.96	172145.8	60916.15			
	glm11		+	+					5	-88804.05	177662	66432.33			
	glm16			+					4	-88838.82	177720.7	66491.09			
	glm12	+	+						4	-102557.44	205158	93928.34			
	glm9	+	+		+				5	-102557.32	205168.5	93938.87			
	glm14	+							3	-102577.05	205186.4	93956.78			
	glm15		+						3	-104001.7	208035.7	96806.08			
	glm17								2	-104020.16	208061.9	96832.23			

Table S4.*Continued.*

	Model	P	R	S	P:R	P:S	R:S	P:R:S	df	logLik	BIC	ΔBIC	P (A):R (I)	P (A):R (I):S (W)	P (A):R (I):S (S)
MNND	**glm0**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**13**	**27785.93**	**-55431.7**	**0**	-0.27	-0.31	0.45
	glm1	+	+	+	+	+	+		11	20957.31	-41796	13635.68			
	glm2	+	+	+		+	+		10	18170.48	-36233.2	19198.55			
	glm3	+	+	+	+		+		9	9400.88	-18704.7	36726.98			
	glm5	+	+	+			+		8	7641.22	-15196.2	40235.53			
	glm8		+	+			+		7	3695.32	-7315.2	48116.55			
	glm4	+	+	+	+	+			9	3681.68	-7266.4	48165.38			
	glm7	+	+	+		+			8	2284.5	-4482.8	50948.96			
	glm10	+		+		+			7	2184.54	-4293.6	51138.09			
	glm6	+	+	+	+				7	-2635.83	5347.1	60778.84			
	glm13	+		+					5	-3794.69	7643.3	63075			
	glm11		+	+					5	-6250.1	12554.1	67985.82			
	glm16			+					4	-6320.19	12683.5	68115.22			
	glm9	+	+		+				5	-25465.13	50984.2	106415.88			
	glm12	+	+						4	-25888.47	51820.1	107251.78			
	glm14	+							3	-25919.42	51871.2	107302.9			
	glm15		+						3	-26926.41	53885.2	109316.88			
	glm17								2	-26956.05	53933.7	109365.38			

Table S4.*Continued.*

	Model	P	R	S	P:R	P:S	R:S	P:R:S	df	logLik	BIC	ΔBIC	P (A):R (I)	P (A):R (I):S (W)	P (A):R (I):S (S)
SDNND	**glm0**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**13**	**9407**	**-18673.9**	**0**	-1.41	1.55	1.5
	glm1	+	+	+	+	+	+		11	-4925.83	9970.2	28644.1			
	glm4	+	+	+	+	+			9	-5015.04	10127.1	28800.97			
	glm3	+	+	+	+		+		9	-7055.76	14208.5	32882.4			
	glm6	+	+	+	+				7	-7137.41	14350.3	33024.14			
	glm2	+	+	+		+	+		10	-7918.62	15945	34618.91			
	glm7	+	+	+		+			8	-7997.39	16081	34754.89			
	glm5	+	+	+			+		8	-9808.41	19703	38376.92			
	glm8		+	+			+		7	-14015.24	28105.9	46779.81			
	glm11		+	+					5	-14076.37	28206.6	46880.5			
	glm10	+		+		+			7	-27324.29	54724	73397.9			
	glm13	+		+					5	-28184.6	56423.1	75096.98			
	glm16			+					4	-30232.85	60508.8	79182.69			
	glm9	+	+		+				5	-53659.09	107372.1	126045.94			
	glm12	+	+						4	-54073.87	108190.9	126864.73			
	glm15		+						3	-54790.12	109612.6	128286.46			
	glm14	+							3	-58075.27	116182.9	134856.74			
	glm17								2	-58682.91	117387.4	136061.24			

Table S5. Mean values and credible intervals (upper and lower intervals within brackets) of each resource’s contribution to primary consumers' diet.

		Coarse detritus		Fine detritus		Autochthonous resources	
		Control	Impact	Control	Impact	Control	Impact
Before	Autumn	0.35 (0.08 - 0.58)	0.46 (0.18 - 0.69)	0.19 (0.04 - 0.35)	0.18 (0.04 - 0.32)	0.46 (0.23 - 0.71)	0.37 (0.14 - 0.65)
	Winter	0.63 (0.50 - 0.74)	0.66 (0.52 - 0.77)	0.11 (0.04 - 0.17)	0.13 (0.06 - 0.21)	0.27 (0.17 - 0.39)	0.22 (0.12 - 0.32)
	Spring	0.52 (0.27 - 0.71)	0.42 (0.23 - 0.61)	0.15 (0.02 - 0.33)	0.17 (0.03 - 0.35)	0.33 (0.16 - 0.55)	0.41 (0.22 - 0.60)
After	Autumn	0.39 (0.17 - 0.56)	0.37 (0.17 - 0.59)	0.19 (0.06 - 0.36)	0.20 (0.02 - 0.41)	0.42 (0.25 - 0.61)	0.43 (0.16 - 0.68)
	Winter	0.63 (0.52 - 0.74)	0.59 (0.47 - 0.70)	0.13 (0.05 - 0.22)	0.12 (0.05 - 0.21)	0.24 (0.14 - 0.36)	0.29 (0.19 - 0.40)
	Spring	0.59 (0.45 - 0.72)	0.62 (0.47 - 0.76)	0.13 (0.05 - 0.21)	0.12 (0.03 - 0.23)	0.28 (0.17 - 0.39)	0.26 (0.14 - 0.38)

Table S6. Linear mixed model results of δ^{15}N for the groups considered with Period, Reach and Season as fixed factors and Sample as random. Values in bold indicate significant differences between factors ($p < 0.05$).

	Basal resources				Primary consumers				Omnivores			
	F	p	Coeff.		F	p	Coeff.		F	p	Coeff.	
Period	0.76	0.385			93.16	**<0.001**	1.33 (A)		69.96	**<0.001**	1.93 (A)	
Reach	7.92	**0.005**	-0.61 (I)		100.55	**<0.001**	0.46 (I)		227.65	**<0.001**	1.48 (I)	
Season	9.97	**<0.001**	-0.95 (W)	-1.58 (S)	202.9	**<0.001**	-1.93 (W)	-2.5 (S)	139.55	**<0.001**	-0.91 (W)	-0.37 (S)
Period:Reach	4.65	**0.032**	1.22 (A:I)		43.65	**<0.001**	2.04 (A:I)		23.74	**<0.001**	1.54 (A:I)	
Period:Season	2.58	0.077			38.13	**<0.001**	-2.17 (A:W)	-0.65 (A:S)	61.91	**<0.001**	-2.2 (A:W)	-2.37 (A:S)
Reach:Season	2.16	0.117			3.02	**0.0497**	-0.38 (I:W)	0.12 (I:S)	6.38	**0.002**	0.02 (I:W)	-1.00 (I:S)
Period:Reach:Season	0.7	0.497			2.89	0.057			7.07	**0.001**	-1.59 (A:I:W)	0.13 (A:I:S)

	Carnivores				Fish			
	F	p	Coeff.		F	p	Coeff.	
Period	32.32	**<0.001**	1.41 (A)		0.12	0.73		
Reach	66.59	**<0.001**	1.45 (I)		68.24	**<0.001**	1.76 (I)	
Season	166.14	**<0.001**	-1.07 (W)	-1.2 (S)	8.82	**<0.001**	1.08 (W)	0.23 (S)
Period:Reach	16.49	**<0.001**	1.79 (A:I)		4.41	**0.037**	-0.14 (A:I)	
Period:Season	24.77	**<0.001**	-1.77 (A:W)	-1.73 (A:S)	6.75	**0.002**	-2.37 (A:W)	-2.54 (A:S)
Reach:Season	15.95	**<0.001**	-1.48 (I:W)	-1.06 (I:S)	0.42	0.658		
Period:Reach:Season	1.08	0.34			1.57	0.212		

Table S7. Linear models and linear mixed model results of $\delta^{15}N$ for the basal resources with Period, Reach and Season as sources of variation (and Sample as random factor for mixes models indicated with an asterisk). Autochthonous resources include biofilm, filamentous green algae and bryophytes. Values in bold indicate significant differences between factors ($p < 0.05$).

	Biofilm			Autochthonous resources *		
	F	p	Coeff.	F	p	Coeff.
Period	26.28	**<0.001**	-0.01 (A)	2.48	0.118	
Reach	36.99	**<0.001**	0.12 (I)	7.22	**0.008**	-0.67 (I)
Season	98.16	**<0.001**	-1.11 (W), -3.30 (S)	10.31	**<0.001**	-1.24 (W), -2.98 (S)
Period:Reach	21.55	**<0.001**	2.01 (A:I)	11.37	**0.001**	1.78 (A:I)
Period:Season	2.23	0.117		4.62	**0.012**	0.33 (A:W), 1.75 (A:S)
Reach:Season	0.36	0.702		1.01	0.369	
Period:Reach:Season	1.12	0.333		0.12	0.886	
	Fine detritus			Coarse detritus *		
	F	p	Coeff.	F	p	Coeff.
Period	4.45	**0.039**	2.17 (A)	2.69	0.103	
Reach	24.17	**<0.001**	0.77 (I)	1.08	0.301	
Season	36.03	**<0.001**	0.59 (W), -0.04 (S)	2.44	0.090	
Period:Reach	6.93	**0.011**	1.41 (A:I)	0.67	0.414	
Period:Season	34.37	**<0.001**	-3.34 (A:W), -3.00 (A:I)	0.54	0.585	
Reach:Season	3.34	**0.043**	0.32 (I:W), -0.97 (I:S)	2.69	0.071	
Period:Reach:Season	0.56	0.576		0.54	0.586	

Table S8. Mean values and credible intervals (upper and lower intervals within brackets) of the iso-space metrics of the consumers (invertebrates and fish).

		CD		MNND	
		Control	Impact	Control	Impact
After	Autumn	1.57 (1.37 - 1.79)	0.92 (0.66 - 1.17)	1.50 (1.23 - 1.79)	0.90 (0.64 - 1.19)
	Spring	1.56 (1.32 - 1.79)	2.02 (1.78 - 2.24)	1.45 (1.16 - 1.72)	1.84 (1.57 - 2.11)
	Winter	2.55 (2.36 - 2.76)	2.58 (2.34 - 2.84)	2.39 (2.12 - 2.66)	2.18 (1.94 - 2.42)
Before	Autumn	1.49 (1.25 - 1.70)	1.74 (1.54 - 1.96)	1.84 (1.53 - 2.18)	1.51 (1.24 - 1.78)
	Spring	1.89 (1.54 - 2.24)	2.22 (2.11 - 2.36)	1.73 (1.43 - 2.03)	1.93 (1.77 - 2.09)
	Winter	2.38 (2.14 - 2.62)	2.66 (2.44 - 2.92)	1.97 (1.74 - 2.20)	2.34 (2.08 - 2.61)
		SDNND		SEA	
		Control	Impact	Control	Impact
After	Autumn	0.49 (0.12 - 0.85)	0.38 (0.00 - 0.76)	7.20 (5.92 - 8.59)	4.37 (3.59 - 5.24)
	Spring	1.27 (0.90 - 1.68)	1.99 (1.64 - 2.36)	6.89 (5.76 - 7.99)	7.56 (6.28 - 8.82)
	Winter	1.92 (1.55 - 2.27)	2.63 (2.26 - 3.01)	9.39 (7.79 - 11.11)	11.17 (9.17 - 13.12)
Before	Autumn	0.28 (0.00 - 0.59)	1.57 (1.21 - 1.93)	10.31 (8.23 - 12.37)	7.90 (6.36 - 9.54)
	Spring	1.47 (0.96 - 2.02)	2.09 (1.70 - 2.49)	6.06 (5.01 - 7.06)	7.76 (6.70 - 8.96)
	Winter	2.12 (1.77 - 2.52)	2.70 (2.32 - 3.06)	10.15 (8.52 - 11.70)	10.53 (8.9 - 12.31)

Figures

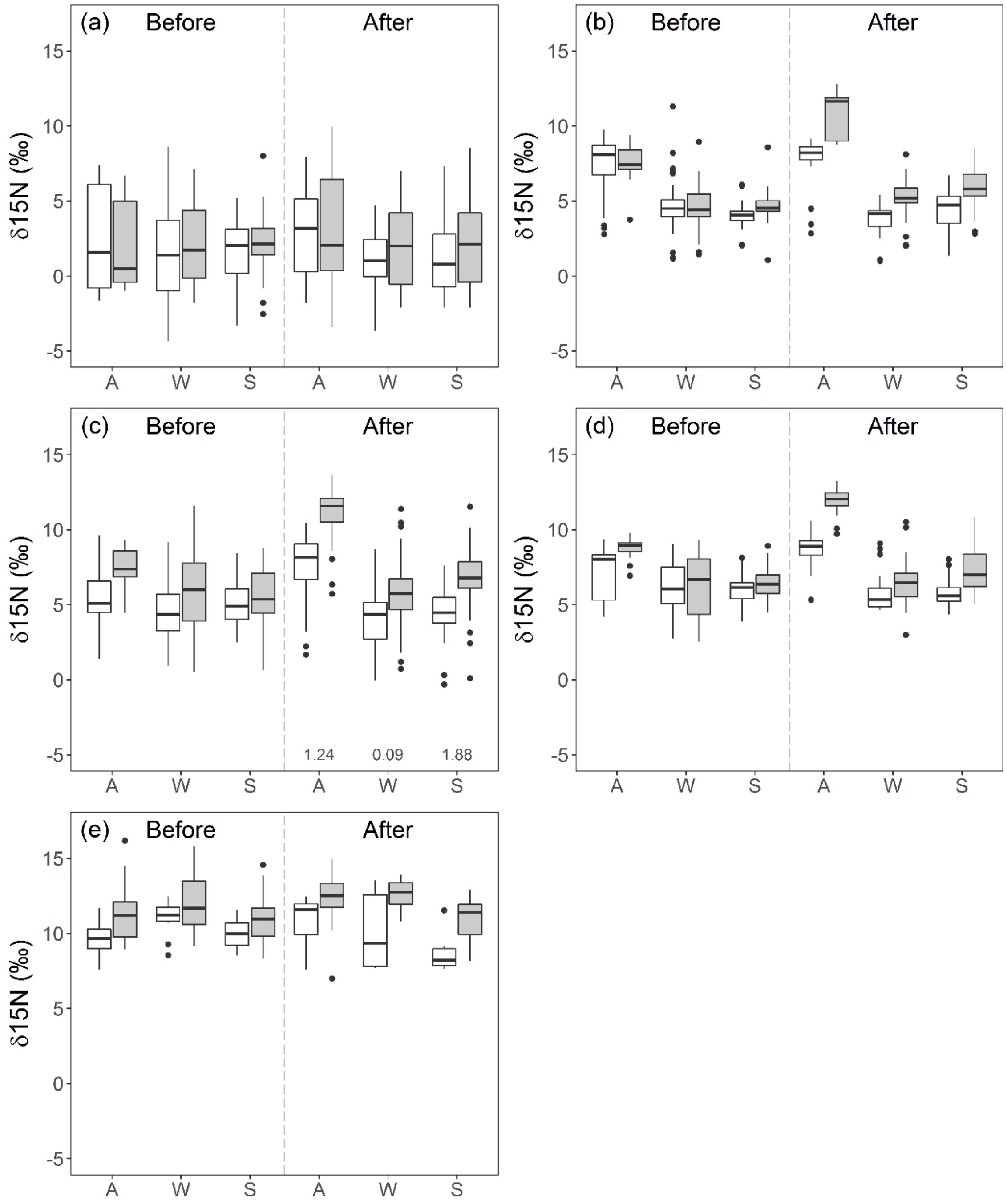

Fig. S1. Nitrogen stable isotope ratios ($\delta^{15}N$, ‰) of (a) basal resources, (b) primary consumers, (c) omnivores, (d) predatory invertebrates and (e) fish in the studied reaches (white for control; grey for diverted) in Autumn (A), Winter (W) and Spring (S) during the Before and After periods. The box plots show the median, the interquartile range, and the tails of the distribution, and dots represent outliers.

Chapter 4: Treated and highly diluted wastewater impacts diversity and energy fluxes of freshwater food webs

Tables

Table S1. Linear model results comparing stock and C:N ratio of basal resources, and characteristics and energy fluxes per trophic group between control (C) and impact (I) reaches before (B) and after (A) the start of effluent addition to assess its effects (BA:CI). Values in bold indicate statistical significance ($p < 0.05$). Coefficients are shown for significant responses and consider B and C as reference in all cases.

			BA			CI			BA:CI	
		F	p	Coeff.	F	p	Coeff.	F	p	Coeff.
Basal resource stock										
	Biofilm	5.56	**0.031**	0.045	0.0004	0.984		3.84	0.068	
	Coarse detritus	2.38	0.133		3.9	0.057	0.617	1.97	0.17	
C:N ratio										
	Biofilm	1.2	0.289		2.21	0.156		2.9	0.107	
	Fine detritus	37.32	**<0.001**	-2.51	25.13	**<0.001**	-1.9	0.28	0.606	
	Coarse detritus	0.13	0.725		2.8	0.104		1.68	0.205	
Trophic group characteristics										
Primary consumers	Mean BM	4.50	**0.034**	0.03	1.51	0.220		1.74	0.187	
	Biomass	4.20	**0.041**	0.28	0.86	0.355		0.93	0.334	
	MR	6.18	**0.013**	0.31	0.52	0.471		1.79	0.181	
Omnivores	Mean BM	0.001	0.946		0.31	0.575		0.18	0.676	
	Biomass	1.18	0.279		1.60	0.207		0.02	0.895	
	MR	1.99	0.160		2.68	0.102		0.001	0.982	
Carnivores	Mean BM	0.76	0.383		0.02	0.878		1.53	0.218	
	Biomass	0.19	0.666		0.96	0.328		1.65	0.200	
	MR	0.16	0.689		1.96	0.163		1.27	0.262	
Energy fluxes per trophic group										
Primary consumers	Herbivory	0.26	0.614		0.61	0.441		0.02	0.901	
	Detritivory	0.16	0.689		20.39	**<0.001**	0.88	58.62	**<0.001**	1.10
	Carnivory	-	-		-	-		-	-	
Omnivores	Herbivory	14.7	**0.001**	0.24	23.44	**<0.001**	0.34	5.47	**0.026**	0.64
	Detritivory	8.13	**0.008**	0.50	29.62	**<0.001**	0.82	0.07	0.797	
	Carnivory	27.62	**<0.001**	0.68	123.38	**<0.001**	0.30	0.01	0.918	
Carnivores	Herbivory	-	-		-	-		-	-	
	Detritivory	-	-		-	-		-	-	
	Carnivory	3.72	0.063		4.28	**0.047**	0.26	2.83	0.103	

Table S2. C:N ratio (mean and SE) of basal resources (biofilm, fine and coarse detritus) before (B) and after (A) the start of effluent addition in control (C) and impact (I) reaches.

	B		A	
	C	I	C	I
Biofilm	6.69 ± 0.10	6.91 ± 0.13	8.64 ± 1.30	6.61 ± 0.31
Fine detritus	15.46 ± 0.27	13.50 ± 0.26	12.94 ± 0.28	10.52 ± 0.76
Coarse detritus	20.60 ± 1.16	18.24 ± 0.36	19.94 ± 0.53	19.60 ± 0.74

Table S3. AFDM in grams (mean and standard deviation) and abundance of fish sampled in the study before (B) and after (A) the start of effluent addition in control (C) and impact (I) reaches.

	B		A	
	C	I	C	I
Anguilla anguilla	5.94 ± 6.20 (14)	4.59 ± 4.46 (13)	16.00 ± 23.80 (11)	7.46 ± 7.66 (21)
Barbatula quignardi	-	0.55 ± 0.37 (83)	1.52 (1)	1.05 ± 0.35 (23)
Phoxinus bigerri	0.86 ± 0.86 (11)	0.63 ± 0.42 (101)	1.02 ± 0.67 (38)	0.55 ± 0.35 (280)
Salmo trutta	18.31 ± 4.59 (4)	11.56 ± 8.62 (15)	2.58 ± 0.62 (3)	5.71 ± 7.40 (5)
Total individuals	29	212	53	329
Total AFDM (g)	165.85	341.66	223.9	363.56

Figures

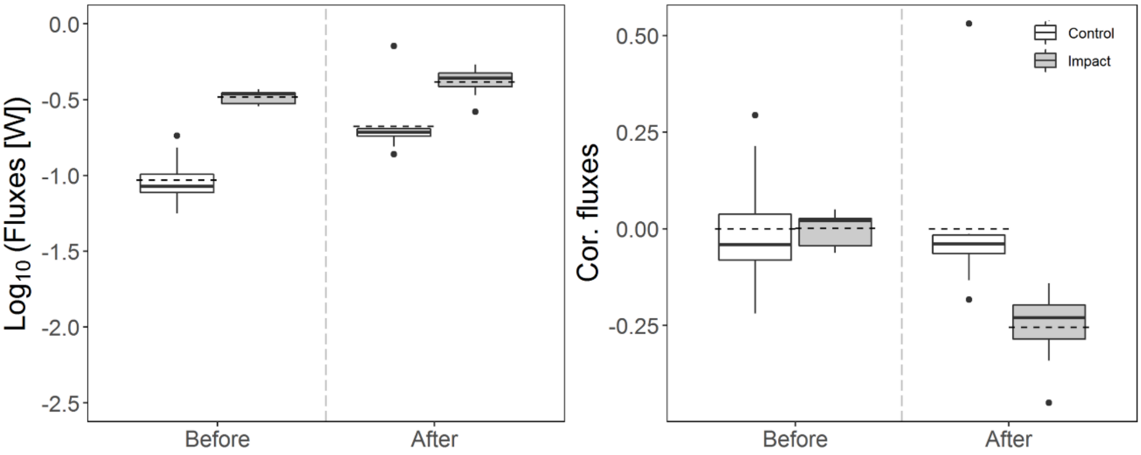

Fig. S1. Explanatory figure to clarify the interpretation of BACI results. The box plots show the median, the interquartile range and the tails of the distribution. Dots represent outliers and dashed lines mean values.. The first plot shows the results regarding total energy fluxes for the periods before and after and the control and impact reaches in this experiment (see Fig. 3). Data for the second plot has been obtained by substracting the intercept to all four cases and the spatial and temporal differences (*BeforeImpact - BeforeControl* and *AfterControl - BeforeControl*) to *Impact* and *After* cases, respectively. This correction allows graphically isolating the effect of the effluent and is aligned with the coefficient for the interaction term in the model (see Table 2).

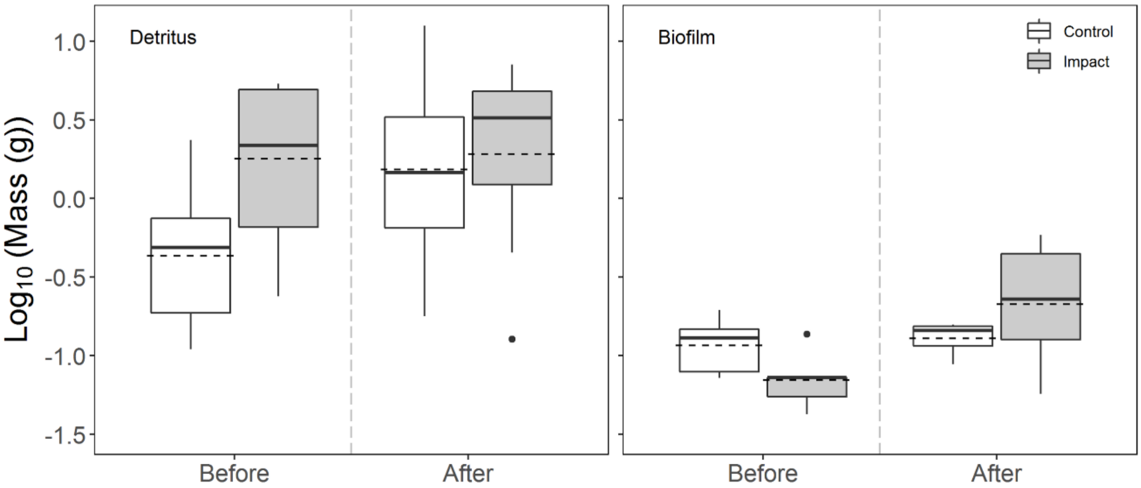

Fig. S2. Detritus and biofilim abundance in the studied reaches before and after the addition of the effluent. The box plots show the median, the interquartile range and the tails of the distribution. Dots represent outliers and dashed lines mean values.

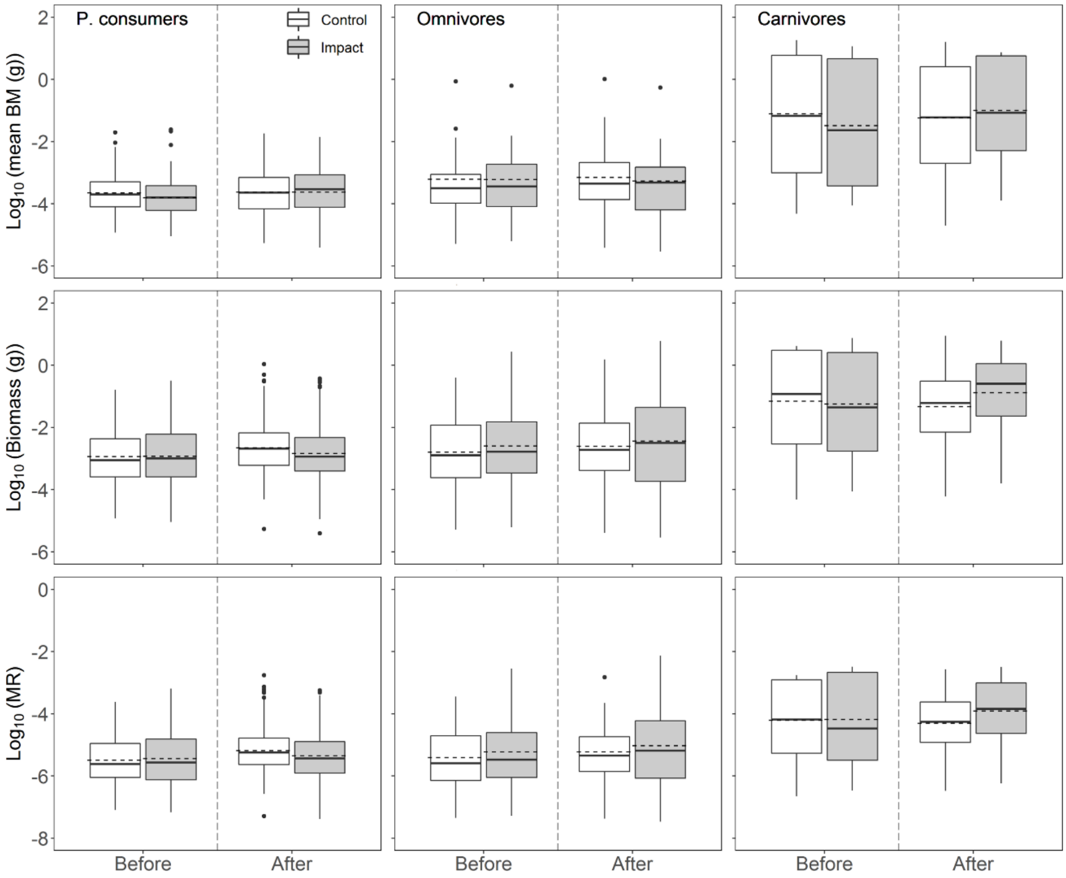

Fig. S3. Mean body mas, biomass and metabolic rate (MR) of primary consumers, omnivores and carnivores respectively. The box plots show the median, the interquartile range and the tails of the distribution. Dots represent outliers and dashed lines mean values.

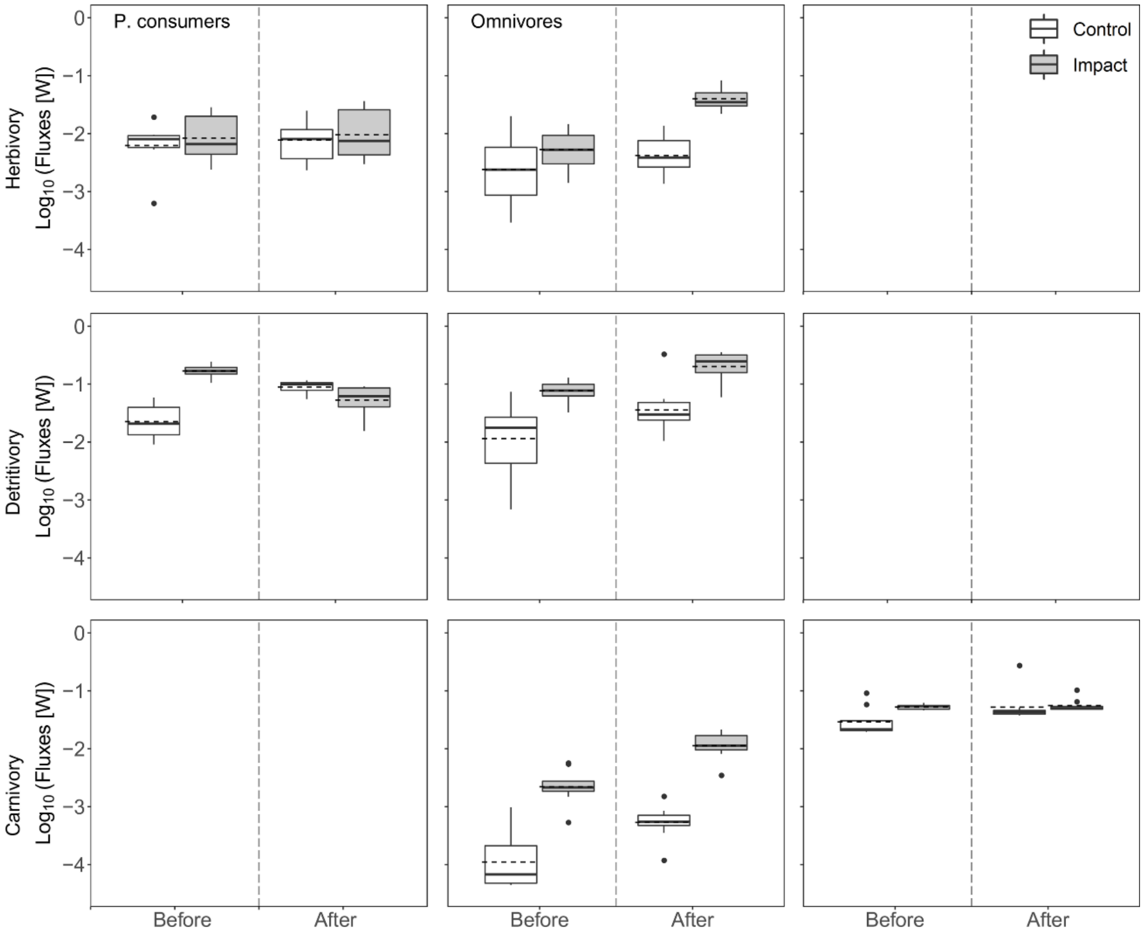

Fig. S4. Energy fluxes to each trophic guild (primary consumers, omnivores, and carnivores) and function (herbivory, detritivory and carnivory). The box plots show the median, the interquartile range and the tails of the distribution. Dots represent outliers and dashed lines mean values.

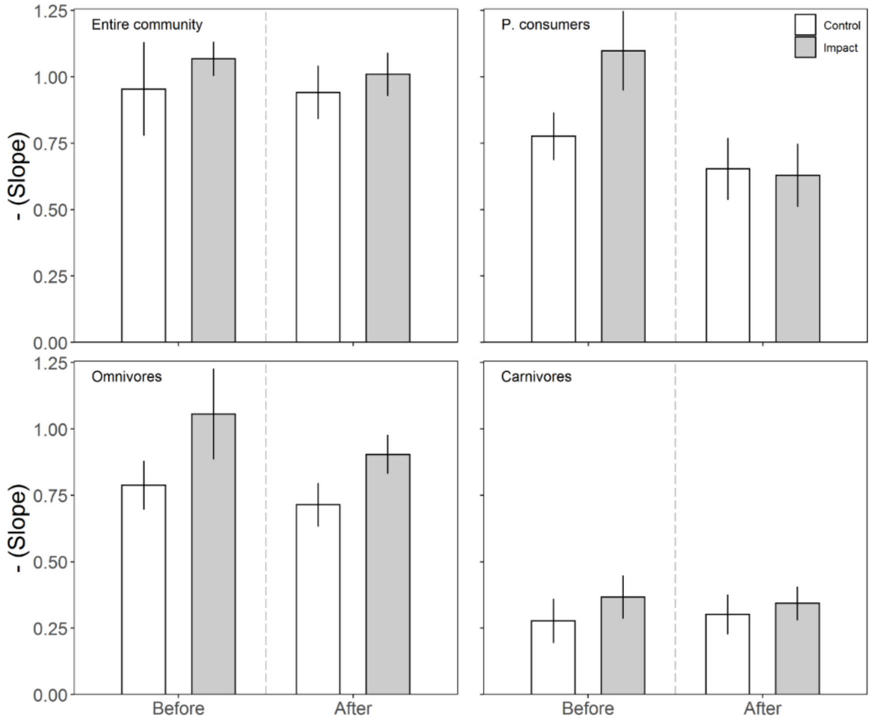

Fig. S5. Slope of the body mass-abundance size spectra for the entire community, primary consumers, omnivores and carnivores. Bars represent the mean and error bars the standard error.

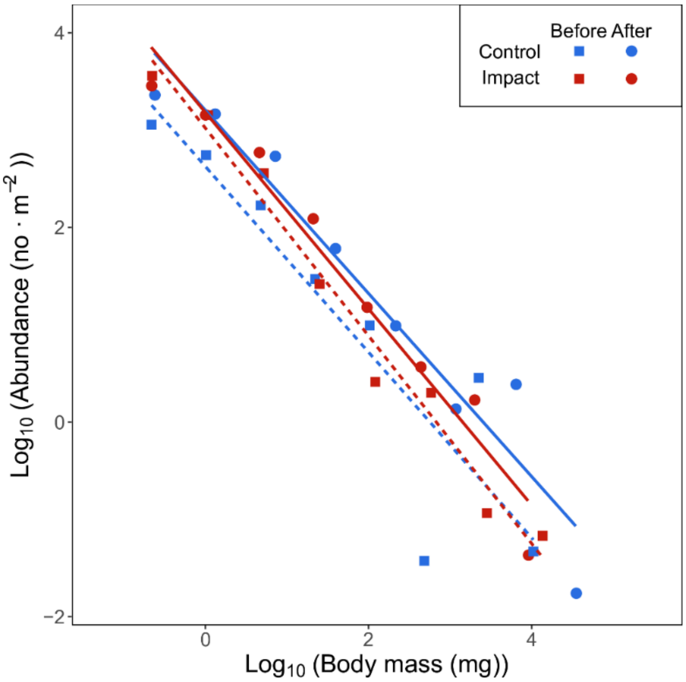

Fig. S6. Size spectra for invertebrates and fish communities before and after the start of the effluent addition in control and impact reaches. Regression lines are derived from the linear models. Before-Control (slope: -0.95 (CI ± 0.43)), Before-Impact (slope: -1.07 (CI ± 0.20)) After-Control (slope: -0.94 (CI ± 0.25)), After-Impact (slope: -1.01 (CI ± 0.16)).